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Pharmacological inhibition of N-MYC and p53 Activation Induces Tumor Immunogenicity in Neuroblastoma

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Pharmacological inhibition of N-MYC and p53 Activation Induces Tumor Immunogenicity in Neuroblastoma

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Contents

Abbreviations

APS	Ammonium persulfate
ATP	Adenosine Triphosphate
Bcl-XL	B-cell lymphoma-extra large
B2M	β2-Microglobulin
CAD	Carbamoyl-Phosphate synthetase 2, Aspartate transcarbamylase, and Dihydroorotase
CDKs	Cyclin-dependent kinases
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
ERAP	Endoplasmic reticulum aminopeptidase 1
FBS	Fetal bovine serum
GLUT1	Glucose Transporter 1
HIF-1α	Hypoxia-inducible factor 1-alpha
HLA	Human leukocyte antigen
HPRT1	Hypoxanthine phosphoribosyltransferase 1
MCL-1	Induced myeloid leukemia cell differentiation protein
MCT4	Monocarboxylate transporter 4
MDM2	Mouse double minute 2 homolog
NB	Neuroblastoma
PBS	Phosphate-buffered saline
PFK1	Phosphofructokinase 1
PFKM	6-phosphofructokinase muscle type
RIPA	Radioimmunoprecipitation assay
RPLP0	Ribosomal protein P0
RPL13A	Ribosomal protein L13a
STAT	Signal transducer and activator of transcription
TAM	Tumor-associated macrophages
TBP	TATA-Box Binding Protein
TEMED	Tetramethylethylenediamine
WNT	Wingless-related integration site

1. Introduction

Neuroblastoma is the most commonextracranial solid tumor occurring in infancy and the second most commonly occurring in children. It is the neoplasm of the sympathetic nervous system. (Park et al., 2008). Neuroblastoma is characterized by the complex framework of genetic irregularities interrelated to determine the clinical phenotype. Neuroblastoma is distinguished by somatically gained genetic events that conduct a result of gene overexpression (oncogenes), gene inactivation (tumor suppressor gene), or modifications in gene expression. N-Myc proto-oncogene is known to be amplified in 20% to 25% of neuroblastomas, which plays multiple roles in malignancy and of maintenance of stemness (Maris & Matthay, 1999). In neuroblastoma, the important tumor suppressor gene TP53 is wild type in most of the patients at diagnosis, through overexpression of its negative regulators (Mdm2 or MDMX) and other unknown mechanisms (Tweddle et al., 2003). The p53 has shown to down-regulate N-Myc at protein level in neuroblastoma (Burmakin et al., 2013).

Therefore, it was tempting to study the combinatorial effects of N-Myc inhibition and p53-activation for complete tumor regression in neuroblastoma, as the high-risk neuroblastoma with N-Myc amplification frequently leads to a fatal clinical outcome even though multimodal therapy, there is a need to develop more targeted strategies by targeting both oncogene (N-Myc) and tumor suppressor (p53). The combination of two anti-cancer treatments can increase efficiency due to targeting different pathways or by acting in a synergetic or additional manner (Mokhtari et al., 2017).

A number of studies have succeeded to find molecules that appear to restore proper tumor suppressor activity of p53 both *in vitro* and *in vivo* (Sanz et al., 2019). Moreover, finding the small molecules to target N-Myc is quite challenging. Recently, small molecules MI-6, which inhibit MYC-MAX (heterodimer important for MYC-driven transcription activation) showed promising *in vitro* response (Castell, Yan, Fawkner, Hydbring, et al., 2018a). Notably, several studies indicate that the N-Myc amplification in neuroblastoma is associated with repressed cellular immunity (P. Zhang et al., 2017). MYC oncogene represses immune surveillance both directly and indirectly in different cancers (Casey et al., 2017, 2018). Several studies suggest that p53 can influence innate immune responses as part of its tumor suppressor activities (Sanz et al., 2019) but the detailed mechanism is still under investigation.

It has been suggested that p53 and Myc inverse regulate several common target genes expression including innate immune genes. In this study, we tried to get a better understanding of the innate immune response by inhibition of N-Myc using MYCMI-6 and activation of p53 using ATSP-7041 and Nutlin-3. Interestingly, monitoring p53 and MYC level, tumor cell expression of endogenous retroviral elements (ERVs) increased which leads to enhanced STAT1 expression, activity and followed by HLA class I expression has been significantly enriched. Our findings suggest that MYC inhibitor and p53 activators increase tumor immunogenicity and provide a rationale for new combination regimens comprising N-Myc inhibitor and p53-activators as an anti-cancer treatment.

2. Review of literature

2.1. Cancer

Cancer, which is defined as indigenous cells, abnormal cells that arise from one's own normal tissues not from cells that were introduced into the body by infection from another person. It is a disease that originates when a single normal body cell undergoes complex and successive genetic and epigenetic aberrations that transforms onto a cancer cell. Through years of proliferation, and uncontrolled growth, this cell and its successors become a population of cells which is recognized as a tumor. Consequently, this tumor builds-up the symptoms of what is known as cancer. Although a tumor is monoclonal in origin; meaning that it was originally acquired from a single ancestral cell, but tumor is like an organ which consists of varying characterized cells including non-cancerous cells as fibroblasts, vascular endothelial cells, pericytes, extracellular matrix and innate and adaptive immune cells.

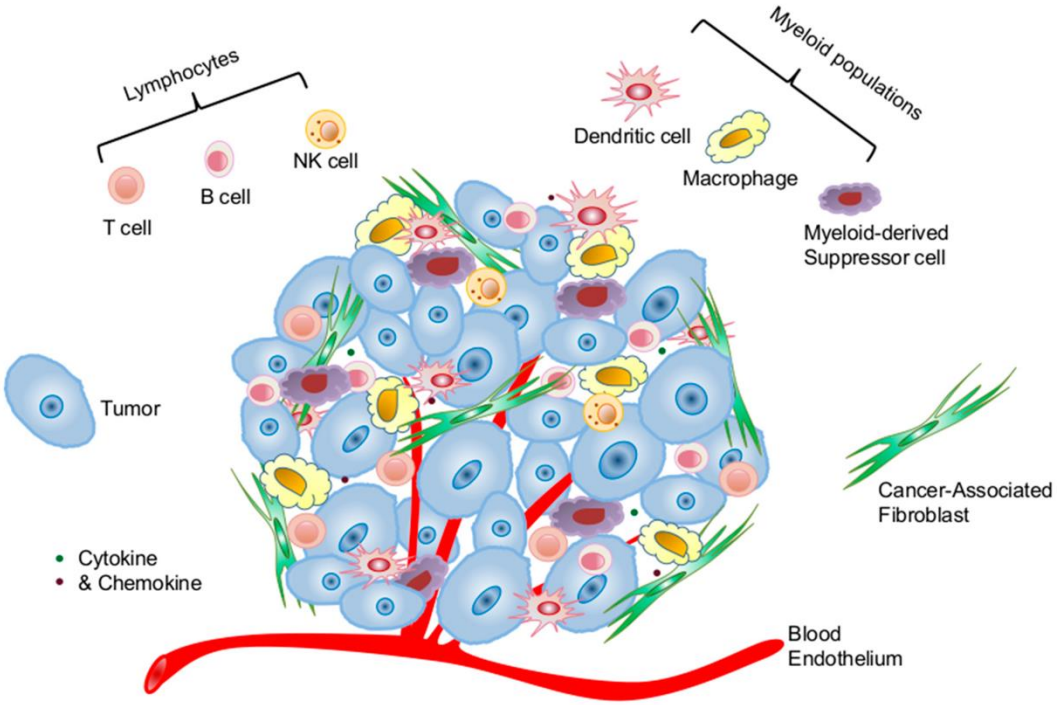


Figure 1: Tumor microenvironment (Cui & Guo, 2016). This figure shows the tumor immunological construction of the tumor microenvironment. It is made up of cellular components and molecular components. The cellular components are comprised of immune cells derived from hematopoietic origin and stromal cells derived from non-hematopoietic origin. The immune cells include tumor-infiltrating T, B, and natural killer cells. It also includes myeloid populations of macrophages, myeloid-derived suppressor cells, and dendritic cells. The stromal cells are comprised of cancer-associated fibroblasts and endothelial cells.

2.2. Hallmarks of cancer

During the successive multi-step process of genetic and epigenetic aberrations that the precancerous cell undergoes, it attains the features that generate the malignant growth of cancer cells, features which enable the cancer cell to survive, grow, proliferate and disperse. Almost every cancer type, if not all, acquire those set of traits that is defined as the hallmarks of cancer. These six include the ability to sustain proliferative signaling, the ability to evade growth suppressors, acquired resistance to cell death, induction of angiogenesis, activation of tissue invasion and metastasis, and uncontrolled unstoppable replication (Hanahan & Weinberg, 2011).

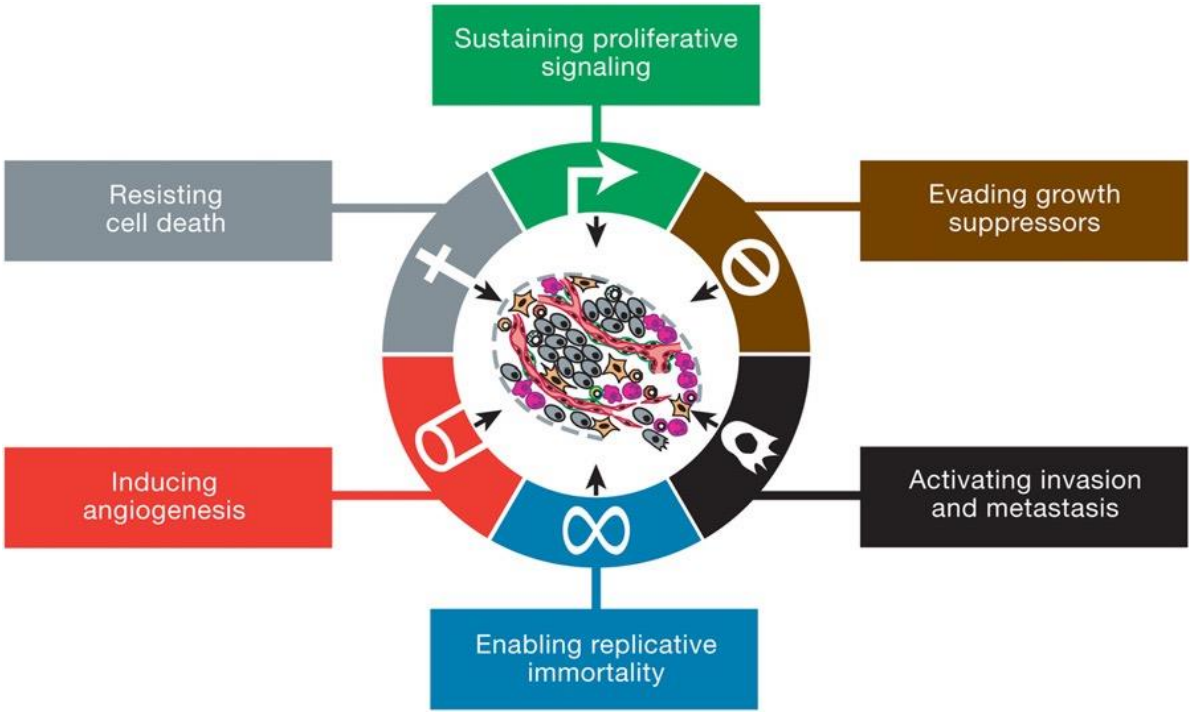


Figure 2: The six hallmarks of cancer and the acquired traits of cancer (Hanahan & Weinberg, 2011)

These six fundamental hallmarks of cancer have been updated with additional two which are believed to play role in the pathogenesis of again some and might be all types of cancer. These two include formatting cellular energetics; consequently, results in genome instability and mutation. The second one is the ability to evade immune surveillance by T and B lymphocytes, macrophages, and natural killer cells; thus, leading to tumor-promoting inflammation (Hanahan & Weinberg, 2011).

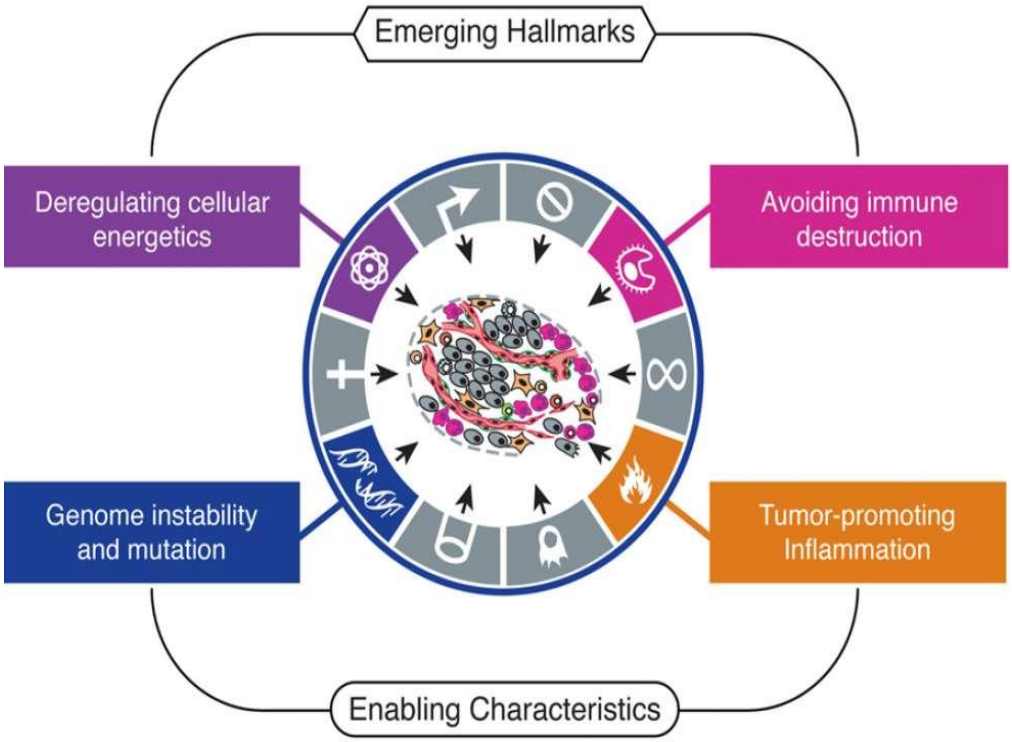


Figure 3: Two additional hallmarks of cancer unfolded (Hanahan & Weinberg, 2011)

2.2.1. Sustaining proliferative signaling

One of the most prominent features of a cancer cell is that it can proliferate continuously and independently, without external stimuli. In a normal cell, there is a tight regulation of both the growth-encouraging and growth-inhibiting factors by mitogenic growth signals, to assure normal cell number, tissue construction and function. However, in cancer cells, this cascade of tight control is disrupted and cells proliferate in despite of proliferative signaling; hence, uncontrollable and unstoppable growth.

In neuroblastoma, the growth is governed by multiple growth factors which communicate with specific receptor tyrosine kinases and non-receptor tyrosine kinases available at cancer cell. Some of these include the neurotrophin receptors, TrkA/NTRK1, TrkB/NTRK2 and TrkC/NTRK3, where they attach to a family of cognate ligands such as nerve growth factor (NGF), brain-derived neurotropic factor (BDNF) and neurotrophin-3 (NT3), respectively (Brodeur et al., 2009). These receptors have various roles on growth and development, TrkA and TrkB have contrast roles for example, where the former curb proliferation when in contrast TrkB encourages proliferation and development. These receptors have a couple of mechanisms that could lead to its activation, as previously mentioned through autocrine or paracrine

activation (Brodeur et al., 2009). It has been reported that numerous neuroblastoma tumors that on-the-spot develop such as ganglioneuroblastoma are well-supplied in Schwann cells, which in turn are bountiful root of NGF and other neutrotrophins that result in immature N-Myc-amplified and non-amplified neuroblastoma cell lines (Ambros et al., 1996; Kwiatkowski et al., 1998). One more thing that conduce a shoddy clinical outcome in neuroblastoma is credited to the cytokine receptor interleukin-6 (IL-6). It is often found vastly expressed in high-risk N-Myc non-amplified human cancers; whereas, the proteins gp130 and gp80 that construct this receptor, are expressed in the majority of human neuroblastoma cell lines, in both the N-Myc-amplified and non-amplified (Ara et al., 2013).

2.2.2. Evading growth suppressors

Cancer cells couple the sustained proliferative signaling and the constitutive stimulation of growth-stimulatory signals, together with evasion of growth suppressors and signals to negatively regulate cell proliferation. This outcome is mostly gained by the action of the tumor suppressor genes. Tumor suppressor genes function differently to restrict cell growth and proliferation. The two most important genes are the retinoblastoma-associated (Rb) and tumor protein 53 (Tp53). They behave as central control nodes which pilot two of the most fundamental cellular regulatory circuits, activation of senescence or growth arrest and apoptotic programs. These genes are frequently mutated or inactivated in all human cancers.

The Rb protein obtains signals extracellularly and intracellularly originated which dictates whether a cell should run through growth-and-division cycle. On the other side, p53 collects inputs from stress, abnormalities and disrupted cell cycle continuation and for as long as the system is not functioning normally yet.

In neuroblastoma, p53 is the main tumor suppressor, however, it is often found wild-type at diagnosis or even at recurrence (Tweddle et al., 2003). Therefore, it is suggested that the variance in MDM2 pathway might be the medium that gives carte blanche to evade growth suppressors (T. Van Maerken et al., 2009).

2.2.3. Enabling replicative immortality

Under normal conditions, healthy cells are only restricted to undergo certain number of cell division cycles where after they face senescence, this phenomenon is known as “Hayflick limit”; in contrast, the tumor cells where they exhibit unrestricted replication to form tumors.

Telomeres, the chromosomes' ends, play key role in restriction of this division, they are multiple repeats of the hexanucleotide "TTAGGG" which preserves the ends of chromosomes from end-to-end fusions (Shay & Wright, 2000).

In neuroblastoma tumors, telomerase enzyme is surpassingly elevated as in most cancers, such elevation is correlated with N-Myc amplification and the unsatisfactory clinical outcome (Hiyama et al., 1995). Recent studies have showed that telomerase activity in neuroblastoma is under regulation by the tumor microenvironment especially by the inflammatory monocytes and macrophages. It has also been proven that the relocation of the microRNA-21 via exosomes from the neuroblastoma cells to these inflammatory monocytes caused dispense of microRNA-155. These microRNA-155 intend the telomeric repeat-binding factor 1 (TERF1) directly in the neuroblastoma cells. TERF1 is an inhibitor of telomerase; hence, telomerase activity is elevated (Challagundla et al., 2015).

2.2.4. Activating invasion and metastasis

In the tumor site, cells experience morphological changes which affect their cell-cell or cell-matrix interplay and in turn aid them successfully get through the first steps of the multistep process of invasion and metastasis. It is a stream of biological changes that permit cancer cells to migrate from its original starting place to new healthy tissues, ensuing intravasation into blood and lymphatic vessels (Fidler, 2003).

In neuroblastoma, the sites for metastasis are the bone marrow, liver and bones in patients with stage IV neuroblastoma (DuBois et al., 1999). Majority of the cell lines acquired from high-risk patients exhibited receptors CXCR4 and CXCR7 which are the receptors for CXCL12 chemokine, or to which is also known as stromal-derived factor-1 (SDF-1). The former receptor is commonly correlated with the more combative undifferentiated tumors; however, the latter receptor CXCR7 often linked and expressed in more differentiated and mature tumors. Metastasis to the liver and lungs is shined out to when the CXCR4 is overly expressed in neuroblastoma, while when it is the CXCR7 that is overly expressed the adrenal gland and the liver are more favored. In case, they are both simultaneously expressed, neuroblastoma dissemination is greatly directed to face the bone marrow (Geminder et al., 2001; Liberman et al., 2012; Russell et al., 2004).

2.2.5. Inducing angiogenesis

Logically, when cancer cells multiply and proliferate, tumor mass and size increases. In consequence, natural and normal diffusion of oxygen and nutrients could be restricted, which are needed for further growth of the tumor. Herein, the need of this hallmark capability; induction of angiogenesis. This process is defined as the formation of new blood vessels from existing vasculature, which maintains constant delivery of nutrients and oxygen to tumor site coupled with disposal of tumor's metabolic wastes and facilitating of hematogenous metastatic process.

In neuroblastoma, angiogenesis and vasculogenesis are induced as in most cancers. However, in neuroblastoma, MYCN induces the level of VEGF (Kang et al., 2008); even though tumor cells are mostly the roots for VEGF, neuroblastoma cells can trigger the production of VEGF through the bone marrow-derived mesenchymal stromal cells (MSC) which encourages the production of osteoblasts via an intracrine pathway (Haduong et al., 2015).

2.2.6. Resisting cell death

It is well established by many studies that programmed cell death, or apoptosis serves as a barricade against tumor advancement. It is normally activated by certain physiological stresses, both by internal and external stimuli that cancer cells endure thorough the process of tumorigenesis. These include signaling imbalance, unrestrained proliferation, and irreversible DNA damage. However, highly malignant cancers can diminish this programmed cell death, and even become resistant to therapy. (Lowe et al., 2004).

When DNA damage occurs via chemotherapeutic agents, apoptosis is initiated through the tumor protein 53 (p53), consequently downstream genes are triggered leading to cell death. Unfortunately, 50% of all human cancers have mutations in the p53 gene (Ferrara, 2009). Mutation of this protein hinders cancer cells more resistant against cellular stresses that would trigger cell death.

In neuroblastoma, defiance of caspase-8 expression through gene silencing or deletion is a prevailing twist contributes to the neuroblastoma's aggressiveness and metastasis. This defiance eventuates the overlooking of the programmed cell death cancer feature. Not only that but, caspase-8 can irreconcilably assist migration and metastasis in neuroblastoma cell lines in way where it is not relying on its degrading or its apoptotic exertion; but relying on the contact with the extracellular matrix (ECM). A complex that is formed between caspase 8, FAK,

calpain2/CPN2 and calpastatin consequent to integrin ligation to ECM proteins interrupts the CPN2/calpastatin binding. Therefore, CPN2 is now activated and strengthening the breakage of focal adhesion substrates, resulting in MYCN amplified and non-amplified human neuroblastoma cell lines to migrate in vitro. (Barbero et al., 2009). Another mechanism that has a hand in resisting cell death is upregulation of anti-apoptotic proteins which aid cell survival in neuroblastoma, this upregulation is credited to the tumor microenvironment. For instance, IL-6 triggers STAT3 encourages the exhibition of various pro-survival proteins such as surviving, MCL-1 and Bcl-XL. (Ara et al., 2013).

2.2.7. Reprogramming energy metabolism

As previously mentioned, cancer cells possess the capability of sustained proliferation, this capability must be complemented with a supply of energy metabolism to fuel this constant proliferation, cell growth and division, and survival in harsh conditions. Normally, cells process glucose aerobically through glycolysis to pyruvate in the cytosol, then finally to carbon dioxide in the mitochondria in what is called Krebs cycle. However, in the absence of free oxygen, that is anaerobically, glycolysis is advantaged with almost no pyruvate relocated to the oxygen-consuming mitochondria. In cancer cells, it has been observed by Otto Warburg that although presence of oxygen, glycolysis is still privileged, that is defined as “aerobic glycolysis”. This phenomenon is largely known as the “Warburg effect” (Hanahan & Weinberg, 2011).

On the grounds that this metabolic switch takes place in cancer cells, there is approximately 18-fold lower efficiency of ATP production supplied by glycolysis in comparison to the conventional mitochondrial oxidative phosphorylation that produces up to 36 ATP for each glycose molecule catabolized. So how can cancer cells counterpoise this deficiency? Upregulation of glucose transporters is one side of the key. Glut1, 2, 3, and 4 are notably upregulated, hence, more glucose uptake. Another way for making up the deficiency is by upregulating the expression of glycolytic enzymes, example of those oncogenes, which are upregulated and manifest extensive induction of cancer, are Ras, Myc, and HIF-1α. Two of those oncogenes, c-Myc and HIF-1α, and under lost or weakened control by p53, other glycolytic enzymes are transactivated directly or indirectly favoring glycolysis. The consensus Myc and HIF-1α binding motifs are abundantly found at the glycolytic gene promoter areas. It has been observed that HIF-1α is majorly working in hypoxia, whereas; c-Myc triggers the

expression of glycolytic genes under normoxia, advantaging the tumor with unceasing glycolysis, consequently; unstoppable proliferation and growth (Phan et al., 2014).
In opposition to that, glucose uptake is abolished directly by inhibition of the transcription of the glucose transporters Glut1 and Glut3 by p53. Furthermore, p53 represses the activation of NF-κB, accordingly; Glut3, which is a target gene of NF-κB is downregulated substantially. (Kawauchi et al., 2008). p53 induced glycolysis regulatory phosphatase (TIGAR) is also another gene its expression is affected positively by p53, to serve as a brake to the cancer glycolysis. TIGAR reconverts fructose 2,6-bisphosphanate which is an allosteric activator of a dominant glycolytic enzyme, PFK1, to fructose 1-phosphate (Bensaad et al., 2006; Bensaad & Vousden, 2007). Concluding the major role of TIGAR hindering the cancer glycolytic flux.

However, p53 is almost always found mutated or malfunctioning in most human cancers.
The possible justifications of why cancer cells favor this glycolytic switch is that more pyruvate is converted to lactate than usual which is a favored pathway as a result of the upregulation of lactate dehydrogenase A (LDHA) (DeBerardinis et al., 2008). Cancer cells favor this pathway due to its generated NADH which serves as an accelerator to the glycolysis. Lactate also has another roe when it is secreted in the tumor microenvironment facilitated by the MCT4 transporter, it feeds other cancer cells, those with shortage of constant nutrient supplies from blood stream. This synergy between both the lactate-producing and the lactate-utilizing tumor cells comes to fruition in terms of the tumor's adaptation to the ongoing changing, chaos and diversity in the tumor microenvironment due to the leaky and scant tumor vasculature (Draoui & Feron, 2011; Semenza, 2008).
Another advantage of this major shunting of pyruvate to lactate is that, less reactive oxygen species are produced, thereupon; tumor survival is enhanced as the level of oxidative stress in the tumor cells is lowered (Heiden et al., 2009; Yeung et al., 2008). Lactate can also switch the pH of extracellular microenvironment to a more acidic one, expediting cancer invasion and metastasis through easing the activity of metalloproteases for destructing extracellular matrix (Bonuccelli et al., 2010; Martinez-Outschoorn et al., 2011).
Another hypothesis of why cancer cells favor this glycolytic switch is that the intermediates of this pathway get involved metabolically into various biosynthetic pathways such as generation of nucleosides and amino acids, thereby; enhanced synthesis of macromolecules and organelles required for constructing new cells. Aside from this metabolic function, glycolytic intermediates exhibit key roles in encouraging cancer survival, metastasis, invasion, chromatin

remodeling, gene expression regulation and many other crucial cellular processes (J. W. Kim & Dang, 2005; Yeung et al., 2008).

In neuroblastoma, there is growing evidence that the Warburgian bias is not a result of a decreasing number of mitochondria. Upon detailed examination, equal levels of mitochondria in human NB samples in comparison to normal kidney tissue were detected (Lyser, 1974). Peculiarly, remarkable decrease in the copy numbers of mtDNA (mitochondrial DNA) was observed, along with inclusive reduced enzyme activity of OXPHOS (Oxidative phosphorylation) in NB samples (Feichtinger et al., 2010).

One of the common chromosomal aberrations seen in advanced-stage NBs is the gain of chromosome 17. This gain results in overexpression of an anti-apoptotic protein BIRC5/Survivin. This protein is known to be enmeshed in this hall mark; reprogramming metabolism in NB. This protein tend to trigger fragmentation of mitochondria and lower mitochondria respiration (Hagenbuchner et al., 2013; Plantaz et al., 1997).

Furthermore, N-Myc; which is amplified notably in half of all high-risk NBs, tend to correlate with the modulation of energy metabolism, either directly or indirectly through governing genes that take role in glycolysis, glutamine metabolism, fatty acids metabolism and mitochondrial function (Ren et al., 2015). This amplification of N-Myc seems to encourage the Warburg effect in NBs, by setting in motion the transcription of a few glycolytic genes (Qing et al., 2010).

2.2.8. Evading immune destruction

The healthy body has an immutable immune surveillance property which its key role is to suppress and abolish formation and advancement of emerging and late-stage tumors, and micrometastases. This means that the cells and tissues are under round-the-clock tight inspection against any formation or upraise of any changes leading to incipient neoplasia due to a failed intrinsic tumor suppressor mechanism. Therefore, this proves that cancer cells possess a capability that makes them evade immune surveillance, in other words; the concept of tumor immunoediting. In alignment with that, higher frequency of certain cancers is seen in immunocompromised human beings or at least more rapid growth of the tumor (Vajdic & Van Leeuwen, 2009). Nevertheless, most of these rising cancers are virally induced, this may give an insight about how decreasing the viral burden might negatively impact formation of these tumors. These findings brought us to the conclusion that, immune surveillance has the capacity to curb development of more than 80% of tumors of nonviral etiology. Immunodeficient mice were constructed to study the frequency of tumor formation and/or rapidness of growth

compared to immunocompetent. It has been shown specifically that loss of function or under developed CD8+ cytotoxic T lymphocytes (CTLs), CD4+ Th1 helper T cells (Th), or natural killer (NK) cells, any of these deficiencies manifested escalated tumor occurrence, with even higher prevalence or more aggressive development, if T cells and NK cells deficiencies were combined (Teng et al., 2008). In general, CTLs and Th cells produce interferons (IFN)-γ and cytotoxins to obstruct cancer development, however; some factors as chronic inflammation can counterbalance this obstruction and ease promotion of cancer (Zamarron & Chen, 2011).

Immunoediting: the concept that explains why cancer can sometimes lie dormant in patients for years before re-emanating or why tumors can bypass or evade a fully functional immune system and proliferate (Dunn et al., 2002). Normally, immune system has the capacity to eradicate vulnerable cancer cells as they present tumor antigens, nevertheless; if genetic instability exists, it aids tumor cells to escape this immune surveillance by continuous tumor proliferation with reduced immunoreactivity. Cancer cells keep on dividing and accumulating mutational changes either through immune-induced inflammation or by chance, and this is counterbalanced by immune eradication, this equilibrium keep the tumor dormant. Nonetheless, eventually tumors gain the ability to halt the eradication by the immune system, by gaining immune suppressive effects or by overlooking the expression of target antigen, this is when the tumor starts to be clinically presented. Furthermore, some immune suppressed patients receiving organ transplants have demonstrated donor-derived cancers, supporting the concept of dormant cancers; whereas, the donor was tumor-free and the cancer cells were silenced in a dormant state, but when transplanted to an immune suppressed patient, cancer cells were triggered (Strauss & Thomas, 2010).

In addition, highly immunogenic cancer cells can escape immune elimination through silencing elements of the immune system that is originally produced or excreted to eradicate them. Cancer cells tend to debilitate CTLs and NK cells by the action of immunosuppressive factors like TGF-β (Lanza et al., 2019; Yang & Weinberg, 2008). Another defense mechanism that cancer cells can execute is employing inflammatory cells which are steadily immunosuppressive, such as regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs), where both can offset the action of CTLs (Mougiakakos et al., 2010; Ostrand-Rosenberg & Sinha, 2009).

Additionally to what's mentioned above, neuroblastoma cell lines normally have depressed levels of peptide presenting HLA class I molecules, meaning that recognition by CTLs is tarnished (Main et al., 1985). Once again, one of numerous roles of the tumor microenvironment is governing the expression of major histocompatibility complex (MHC) I

in neuroblastoma cell lines. The interferons generated by NK cells and T cells intensify the expression of MHC-I (Handgretinger et al., 1989; Lampson & Fisher, 1984). Also, NTRK1/TrkA when stimulated through bounding to NGF results in a heightened immunogenicity by overexpression of MHC-I (Pajtler et al., 2013); however, MYCN amplification, which is common in neuroblastoma, elicit repression of MHC-I (Bernards et al., 1986a). Previous studies have demonstrated evidence that, MYCN encompass paracrine regulative capacity on the immune cells found in the tumor microenvironment, N-Myc represses CCL2/MCP-1 (macrophage chemoattractant protein) which magnetize NKT cells in tumors (Metelitsa et al., 2004; Song et al., 2007a).

Cancer cells also tend to induce a pro-tumorigenic inflammation. An inflammatory environment is initiated and mediated through the innate immune cells such as macrophages, monocytes and neutrophils found in the neuroblastoma cells, resulting in further tumor development and metastasis. It has been previously proven that neuroblastoma cells brainwash TAM to face a T-helper 2 mediated phenotype which reinforce tumor progression and development. Hence, when macrophage is detected in neuroblastoma tumors, it is correlated to an inferior outcome (Asgharzadeh et al., 2012; Larsson et al., 2015).

2.3. Proto-oncogenes and tumor suppressor genes

Two classes of genes have key role in either triggering or opposing cancer. These two are proto-oncogenes and tumor suppressor genes. In a normal healthy cell, these two classes of genes together synchronize normal growth, which assures each tissue and organ is within the normal dimensions and structure for a healthy functioning body. When a mutation occurs, proto-oncogenes are transformed to oncogenes, which triggers and encourages uncontrolled and immoderate growth. In alignment, when tumor-suppressor genes are mutated, their control of this immoderate division is disrupted, both of these mutations lead to overall increased growth and cell proliferation resulting in cancer (Understanding Cancer - NIH Curriculum Supplement Series - NCBI Bookshelf, n.d.).

2.3.1. Proto-oncogenes and oncogenes

Mutations that occur in proto-oncogenes are often dominant in nature meaning that only one of the two alleles if sufficient for induction of cancer, which is when mutated is defined as an

oncogene. Proto-oncogenes typically encode proteins with prominent impact on cells such as activation of cell division, cell differentiation repression, and constraining apoptosis. This impact is critical for normal human development and for the homeostasis of tissues and organs, and typically turned-off once the developmental process under regulation is attained. When proto-oncogenes are mutated and become oncogenes, those proteins with such powerful impact on the development are produced in abundance; bringing forth marked increase in cell proliferation, declined cell differentiation together with halting cell apoptosis, hence; they are not turned off and cancers may eventuate (Proto-oncogenes to Oncogenes to Cancer | Learn Science at Scitable, n.d.). In view of their phenotypes which define cancer, they are oftentimes found mutated or immensely overexpressed in tumor cells.

The alteration or the changeover of a proto-oncogene into an oncogene can be attained through at least three processes, in general it includes a gain-of-function mutation. First mechanism is through point mutation in a proto-oncogene, subsequently; constitutive activation of the protein encoded. This mechanism yields an oncogene that on a small scale different from the encoded corresponding proto-oncogene. Secondly, overexpression of the protein product through gene amplification, or local reduplication of a certain DNA segment that carries the corresponding proto-oncogene. Thirdly, when a chromosome of a proto-oncogene gets translocated to a different chromosomal site and falls under the regulation of a new promoter with higher activity, causing, higher expression or the production of a fusion protein with oncogenic activity. The latter two mechanisms produce oncogenes typical to their proto-oncogenes, however; their oncogenic effect is a result of extremely elevated than normal expression level, or due to being expressed in cells, where under normal conditions not expressed there (Proto-Oncogenes and Tumor-Suppressor Genes - Molecular Cell Biology - NCBI Bookshelf, n.d.).

2.3.1.1. The MYC family

One example of the protooncogenes are the MYC cellular oncogene family, which is made up of 3 well-defined genes, MYCL which encodes L-Myc, N-Myc which encodes N-Myc, and c-MYC which encodes c-Myc or Myc. The role of L-Myc is not fully discovered; however, N-Myc is expressed only in neuronal tissues and is restricted to certain stages of embryogenesis, pre B-cell development, kidney, forebrain, hindbrain and intestine, and it is able to displace Myc in murine development (Malynn et al., 2000).

MYC was first discovered through the studying of fulminant chicken tumors due to oncogenic retroviruses. It was discovered as a viral oncogene (v-MYC) that provokes **my**elo**c**ytomatosis (leukemia and sarcoma).(Duesberg & Vogt, 1979; Sheiness & Bishop, 1979). Its cellular homologue was later identified and obtained from chicken DNA (Vennstrom et al., 1982). According to further research, MYC is persistently remodeled by balanced chromosomal translocation in Burkitt lymphoma, which defined MYC as a bona fide human oncogene.(Dalla-Favera et al., 1982; Taub et al., 1982). MYC is also constantly translocated in multiple myeloma, and found to be markedly amplified in different human cancers, making MYC alterations a constant key finding on the road to cancer (Beroukhim et al., 2010; Shou et al., 2000).

Myc has a critical role in tumorigenesis, cell proliferation and development. It has been found always highly elevated in dividing cells during development as well as in adult tissues. In other words, it is consistently expressed thorough the cell cycle of a proliferating cell, and represses to a minimum during quiescence (Depinho et al., 1987)

Two of the Myc polypeptides created by the MYC mRNA are; one that commences at a CUG upstream of the canonical start codon AUG, and the second one commences at an internal AUG (Blackwood et al., 1994). The canonical AUG translates Myc protein with three domains: a N-terminal transcriptional regulatory domain, a nuclear localization domain and a C-terminal DNA binding domain includes a basic helix-loop-helix-leucine zipper (bHLH-LZ) dimerization motif. Myc and MAX form a dimer to bind to the DNA sequence 5'-CACGTG-3'or alike sequence defined as E-boxes or enhancer boxes. Consequently, transcription activation takes place, and this is when Myc features numerous of its functions. Myc binding to Max is crucial for Myc transforming scheme, as Myc homodimers are idle. This complex formed between Myc and Max appears to displace activators and recruit repressors, hence; repression of some genes (Amati et al., 1993; Blackwood & Eisenman, 1991; Eilers & Eisenman, 2008; Kretzner et al., 1992). The Myc polypeptide commencing at the upstream CUG has an unknown role, while, the shorter one commencing from an internal AUG seems to exhibit a role in stress response and might serve as a dominant negative Myc protein (Hann et al., 1992; Spotts et al., 1997).

Furthermore, Myc also seems to employ DNA replication licensing factors to catalyze DNA replication and it has been studied that Myc regulates the expression of up to collectively 15% of all genes. However, it is still unknown whether its transcriptional activity at replication origins is part-and parcel of its DNA replication function (Dominguez-Sola et al., 2007).

How MYC proto-oncogene is regulated? It is tightly regulated on both the transcriptional and the translational levels in non-transformed cells. MYC is regulated by a range of downstream transcription factors of the Wnt pathway such as CNBP, FBP and TCF. Also, it is governed by non-B DNA structures. Abnormalities in the expression of Myc is often due to deregulated upstream
signaling, or due to amplification or translocation of the MYC gene (Boxer & Dang, 2001; Levens, 2010). The Myc protein has a half-life of about 15-20 minutes, which is a noticeably short half-life. Its transcriptional activity is synchronized by phosphorylation at Ser-62 followed by Thr-58, lastly followed by proteasomal degradation after carrying-out its function (Salghetti et al., 1999). Mutations at the Myc residues Thr-58 and Ser-62 could promote tumorigenesis through stabilizing of the mutant proteins and sustaining its level (Salghetti et al., 1999; Thomas & Tansey, 2011; X. Wang et al., 2011).

MYC is known to be one of the most potent oncogenes that affects multiple cellular processes. MYC activation can't single-handedly initiate tumorigenesis, even though it is one of the most frequently activated oncogenes involved in the pathogenesis of human cancers, MYC overexpression can't trigger neoplastic changes on its own, however; its overexpression can be highly fatal and carry damaging impact on normal cells, peaking proliferative arrest, senescence and/or apoptosis. In other words, Myc pathways evokes cell suicide and cancer (Hoffman & Liebermann, 2008; Nilsson & Cleveland, n.d.). This destructive impact of MYC overexpression in normal cells also relies on epigenetic and genetic contexts. Additionally, the loss of the tumor suppressor p53 coordinate with MYC to trigger cellular growth and tumorigenesis. In conclusion, cellular conditions and specific genetic aberrations can facilitate MYC's capability to more readily initiate proliferation and tumorigenesis.

Myc is a pleotropic transcription factor as explained before that affects multiple cellular process, it is the downstream target of variable mitogenic pathways. It can force the cell cycle advancement in a response to proliferative signals, not only that, but it can actually force cells to re-enter the cell cycle. Myc exhibits such controlling effect on the cell cycle as some of the crucial positive cell cycle regulators are encoded by genes targeted by Myc ex. cyclins, CDKs and E2F transcription factors. Additional to its direct transcriptional activity, it can over activate the CDK inhibitor p27 which halts cell cycle progression, p21 is another protein that is inhibited by Myc. Therefore, tumor suppressing action by both p53 and p21 is manipulated (Bretones et al., 2015; Obaya et al., 1999; Wu et al., 2003).

As discussed before that cancer is a multistep process, which means cells gain a series of mutations that altogether enhance the activity of proto-oncogenes and represses the tumor suppressor gene functions. This results in a heterogenous unleashed tumor cell population proliferating, ignoring inhibitor signals. It has been studied that cancer cells depend greatly on specific oncogenic mutations for their survival and growth. This is defined as "oncogene addiction" (Weinstein & Joe, 2006). This could be explained by the fact that these oncogenes frequently have multiplex role in the pathways regulating proliferation and survival of cells and initially gave hand in tumor phenotype, this make them regarded as the "Achilles' heel" of cancer cells (Sharma & Settleman, 2007; Weinstein & Joe, 2006).

In neuroblastoma, amplification of N-Myc is seen in almost 20%-25% of cases. When N-Myc is amplified, it is often interlinked with high-risk disease and poor prognosis, it is also used as the most distinctive genetic marker of risk in neuroblastoma when found. Structurally, both MYC and N-Myc are highly homologous in the encoding regions. They also both heterodimerize with MAX at consensus E-box sequences. Biologically, N-Myc and MYC are remarkably similar in the aspect of encouraging transformation in rat embryo fibroblasts and triggering of proliferation and cell cycle advancement in quiescent fibroblasts (Huang & Weiss, n.d.). Furthermore, when a deletion of either MYC or N-Myc occurs; normal morphology is still achieved, and no irregular proliferation or differentiation compared to the normal state is observed. This is probably because MYC and N-Myc can compensate for each other (Huang & Weiss, n.d.).
N-Myc over expression is often correlated with invasive and metastatic behavior, as it is involved in all facets of metastasis; adhesion, motility, invasion and break down of surrounding matrices (Huang & Weiss, n.d.).

MYC also impacts immunesurveillance by regulating antigens expression tumor cells. One example of an antigen repressed by N-Myc is monocyte chemoattractant protein-1/CC chemokine ligand 2 (MCP-1/CCL2), essential for chemoattraction of natural kilter T-cell (NKT). When N-Myc is knocked down in N-Myc amplified neuroblastoma cell lines, MCP-1 was revived as well as NKT cell chemoattraction. N-Myc possibly binds to the E-box element of the MCP-1 promoter to obstruct the expression of the chemokine (Huang & Weiss, n.d.; Song et al., 2007b).

2.3.2. Tumor suppressor genes

Tumor suppressor genes or what are known as antioncogenes, are genes which code for proteins that obstruct cell proliferation or survival, hence, halting tumor development. However; these genes are frequently mutated or inactivated in tumors (Weinberg, 1994). One of the most important tumor suppressor genes is the "guardian of the genome" tumor protein p53 is a transcription factor and plays crucial role in controlling cell life and death by governing cell cycle arrest, senescence, apoptosis, DNA repair and metabolism.

2.3.2.2. Tumor suppressor protein p53

TP53 is located at chromosome 17 (17p13.1). p53 has numerous and vital roles in cells, the p53 network is depicted in **figure 4.** The multiple functions of p53 are connected to its ability to take control over specific sets of its variable target genes. Apart from its role in initiation of cell cycle arrest, senescence and apoptosis, p53 is involved in numerous non-canonical pathways, such as tissue remodeling, autophagy, metabolism and the control of reactive oxygen species (ROS). Seeing how complex and intertwined p53'network is, it is noted that the impact of p53 and the impact on p53 are immensely flexible and relies on a collection of factors such as the cell type, differentiation state, stresses and influences from the cellular environment (Kastenhuber & Lowe, 2017).

In normal conditions, TP53 gene is regularly transcribed and translated but the protein is then degraded by the ubiquitin-dependent system in proteasomes. The degradation of p53 is carried out primarily by Mdm2. Mdm2 can also encourage NEDD8 modification of p53, which in turn obstructs its transcriptional activity (Xirodimas et al., 2004). Nonetheless, p53 is activated stabilized via a course of post-translational modifications as a response to a multiple stress signals including DNA damage, oncogene activation, and hypoxia (Giaccia & Kastan, 1998). $P14^{ARF}$ is a tumor suppressor protein and it negatively regulates MDM2. Altogether, MDM2, p53 and $P14^{ARF}$ construct an autoregulatory loop which is often deregulated in human Unfortunately, p53 is found to be defective or inactive in almost all tumors. However, in neuroblastoma, p53 is predominantly wild-type, nevertheless; it has been reported that p53 is often inactivated through MDM2 amplification or $P14^{ARF}$ irregularities or deletions (Chen & Tweddle, 2015). Furthermore, another proposed mechanism for the possible reasoning of non-functioning p53 in neuroblastoma although the absence of mutations in most cases is through the cytoplasmic sequestration such as shielding of the C-terminal nuclear localization signal of

p53, defiance against degradation by MDM2, and the possible communication with glucocorticoid receptors via binding of BK virus of the polyomavirus family. Another factor that might be contributing is that it has been found that conceivably the N-Myc overexpression often found in neuroblastoma masks the p21 mediated blockage of the G1 checkpoint resulting in unobstructed G1 furtherance (Tweddle et al., 2003). Therefore, there is need for a targeted therapies to activate wild-type p53 using p53-Mdm2 antagonists alone or in combination with other small molecules to potentially boost survival rate.

2.4. Small molecules used in our study

In our study, we are using three different compounds, MYCMI-6, Nutlin-3 and Atsp7041. MYCMI-6 is a powerful selective inhibitor of MYC:MAX interaction in cells (Castell, Yan, Fawkner, Hydbring, et al., 2018a). It blocks MYC-driven transcription and binds selectively to the MYC bHLHZip domain, therefore; it blocks tumor cell growth in a MYC-dependent manner, as well as MYC:MAX interaction, represses proliferation and triggers extensive apoptosis in tumor tissue in a MYC-driven xenograft tumor model without implying serious side effects (Castell, Yan, Fawkner, Hydbring, et al., 2018b). Nutlin-3 is a characterized MDM2 inhibitor small molecule which hinder the p53-binding domain of MDM2, hence; p53 is stabilized and activated (Nutlin 3 - an overview | ScienceDirect Topics, n.d.). ATSP-7041 is a stapled α-helical short peptide that potently and selectively dual inhibitor of MDM2 and MDMX. It effectively stimulates the p53 pathway in tumors both *in vivo* and *in vitro* (Chang et al., 2013b).

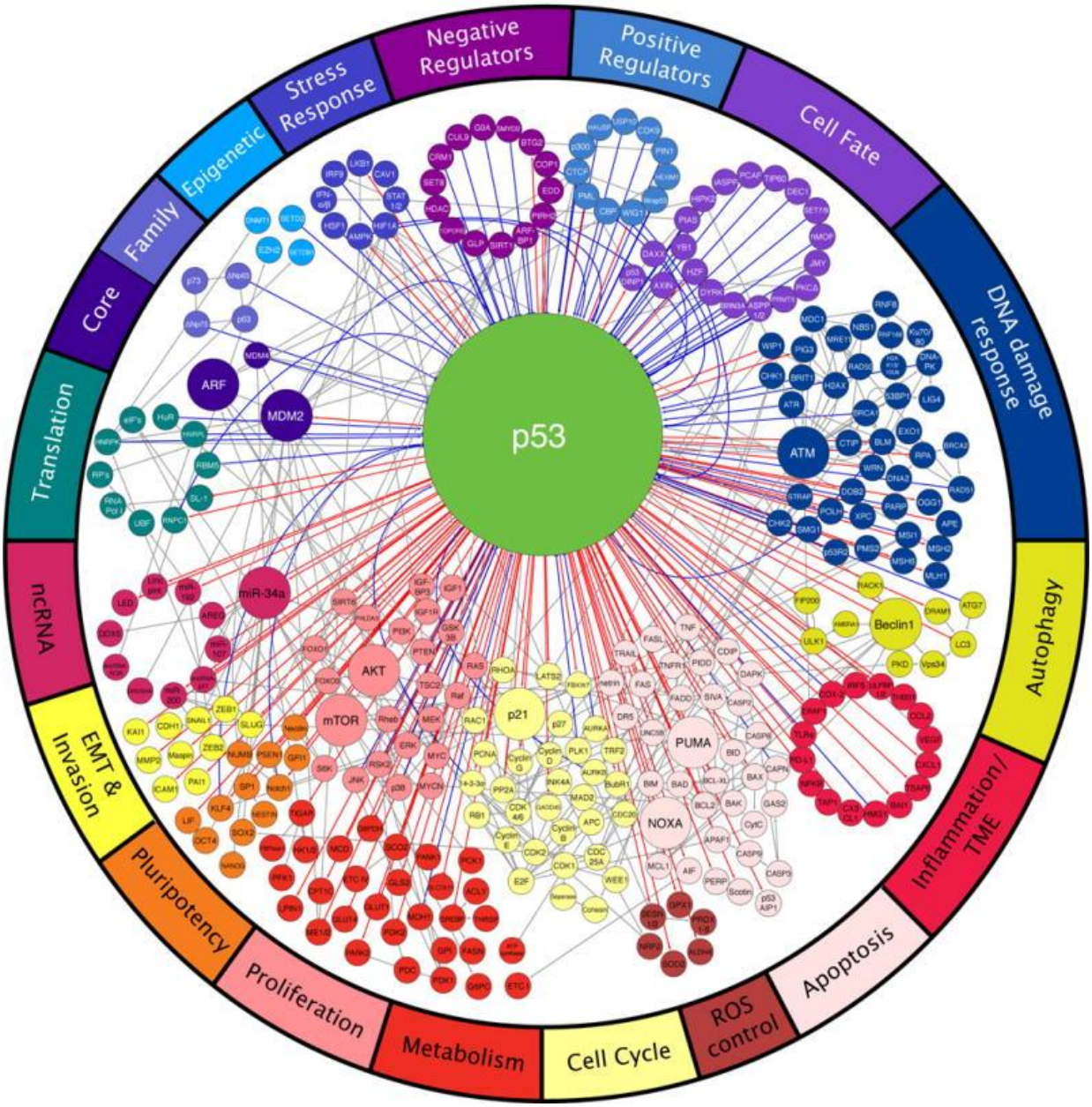


Figure 4: Interactions within the p53 network. Both canonical and non-canonical pathways are shown (Kastenhuber & Lowe, 2017)

3. Aim of the project

Neuroblastoma is the most common extracranial solid tumor occurring in infants and children comprising around 8%-10% of all childhood tumors. Despite intensive multimodal therapy, high-risk neuroblastoma leads to fatal clinical outcome, around 15% of all deaths due to neuroblastoma is in the pediatric sector (Park et al., 2010). Therefore, there is a powerful necessity to identify and establish novel targeted strategies that modulate genes with central roles in tumor progression.

In our project, we are trying to investigate and showcase the effect of molecules that inhibit MYC and activate p53 on activation of the tumor immunogenicity, hence, tumor regression in neuroblastoma. Neuroblastoma cells exhibit tumor-associated antigens however, it has deficiency in the expression of costimulatory molecules and HLA class-I and II molecules (Prigione et al., 2004). Therefore, neuroblastoma cells are seemingly overlooked by the host T-cell compartment, due to the insufficient expression of HLA and costimulatory molecules on antigen presenting cells, which in turn are crucial for effective peptide presentation to T cells (Prigione et al., 2004). In this study, we tried to get a better understanding of the innate immune response by inhibition of N-Myc using MYCMI-6 and activation of p53 using ATSP-7041 and Nutlin-3.

We focused on Atsp-7041 p53-activating and MYCMI-6 MYC-inhibitor small molecules to study the changes they embody on the tumor immunogenicity cascade in neuroblastoma cell lines, possibly leading as a result to tumor regression.

4. Materials and Methods

4.1. Cell Culture

The Cell lines used in my experiments were provided by Dr. Madhurendra Singh and Dr. Ali Rihani. All cell lines except IMR-32 were cultured and maintained in Dulbecco's modified Eagle medium/high glucose (DMEM; Gibco). The cell line IMR-32 was maintained in RPMI-1640 (Hyclone). Both mediums were supplemented with 10% v/v fetal bovine serum (FBS; Thermo Scientific) and 100µg/mL penicillin/streptomycin (Thermo Scientific) referred as complete media, the cells were incubated at 37°C with 5% CO_2. Contaminations with mycoplasma were frequently tested.

Table 1. Materials used in cell culture

Component	Source	Catalogue number
DMEM (High glucose)	Gibco	41965-039
RPMI-1640	Hyclone	SH30027.01
FBS	Thermo Fischer scientific	
PBS	Gibco	
Trypsin-EDTA	Sigma-Aldrich	
DMSO	Sigma	D8418
Resazurin	Sigma	R7017-6

Table 2. Cell lines used (Tom Van Maerken et al., 2011)

Cell line name	Properties
SK-N-FI	P53: mutant MYCN: non-amplified
SKNDZ	P53: mutant MYCN: amplified
SK-N-BE(2c)	P53: mutant MYCN: amplified
IMR-32	P53: wild type MYCN: amplified

SH-SY5Y	P53: wild type MYCN: non-amplified
SK-N-AS	P53: mutant MYCN: non-amplified C-MYC gain

4.2. Cell Passage

Cells were passaged when reaching 70-80% confluency. The cells were washed, one time with sterile 1xPBS, then incubated in 1X Trypsin-EDTA for 2-3 minutes at 37°C incubator. After thorough checking under the microscope to ensure detachment of the cells, trypsin was inactivated using 9 mL of complete medium.

4.3. Cell Counting

A sample of the cells is thoroughly mixed with 0.4% Trypan Blue (Life technologies) in ratio of 1:1, and 10µL transferred to a hemocytometer counting chamber, then counted manually. The required number of cells were then cultured again in fresh plates, already supplied with fresh media. For the 6-well plates, we have used 100,000-200,000 cells per well in 2mL. For the 6-cm plates, 0.5 million cells per plate in 3mL were seeded, and for the 96-well plates, 5000 cells per well in 0.1 mL complete media.

4.4. Compounds treatment

For qPCR and Western blot, one day before treatment cells were seeded in either 6-well or 6-cm dish. The concentration was used as IC50 of each compound determined by resazurin assay.

Table 3. Conditions of treatment and combinations

Conditions	Compounds	Concentration (in µM)
1	DMSO (control)	0.1%
2	MI-6	2
3	ATSP-7041	1
4	MI-6 & 7041	2 + 1
5	Nutlin-3	1

6	MI-6 & Nutlin-3	2 + 1

The cells were treated at two time points, 8 hours and 16 hours under the same conditions in the incubator.

4.5. Cell Viability using Resazurin Assay

Resazurin is a cell permeable redox indicator that was used to examine the viability of the cells following compounds. Since, viable cells with active metabolism can reduce resazurin into the pink and fluorescent resorufin product, and the quantity of the produced resorufin is proportionate with the viable cells. Hence, the most convenient concentration of compounds with a minimal side effects on cell viability can be determined, after quantifying of the resorufin using a microplate fluorometer equipped with a 560 nm excitation / 590 nm emission filter. The assay was performed in 96-well plates. On the following day, the cells were treated with the compounds in two-fold serial dilution as mentioned in figure legends. At the corresponding treatment time point, the existing media is supplemented with 5µM final concentration of resazurin in complete medium. After two hours of incubation, the fluorescence intensity was quantified and IC_{50} for each treatment was determined using Microsoft Excel 2007.

4.6. RNA extraction and Complementary DNA synthesis (cDNA)

4.6.1. RNA extraction using TRIzol reagent (Invitrogen, catalogue number: 15596026)

TRIzol reagent is a monophasic solution of phenol, guanidine isothiocyanate, and other proprietary components which enable the isolation of various RNA with variable sizes.
Isolation of RNA was performed using this method, to isolate the small RNAs. The protocol followed was supplied by Invitrogen for the TRIzol reagent. Before harvesting, the treated cells were washed with 1X PBS and 400 µL of TRIzol was added in each well and cell lysates were collected by scraping and total RNA was isolated according to standard procedure.

Table 4. Concentrations of treatment

Compound	**Concentration µM**
MI-6	2
7041	1

Nutlin	1

DMSO was used as a control, its concentration was set based on the highest volume of combination treatment used. Before harvesting, the old media was removed, and the cells were washed with ice-cold PBS, which was also removed.
A cell scraper was used to collect cells from the 6-well plate using the TRIzol reagent.

4.6.2. RNA extraction using Bio-Rad kit (Catalogue number: 732-6820)

Another method used for RNA extraction was the Aurum Total RNA Mini kit by Bio-Rad.
Cells were seeded and treated as mentioned previously. The adherent cell cultures were harvested using lysis solution (supplemented with 1% ß-mercaptoethanol) into provided tubes, the cells were left with the lysis solution on the shaker for 15 mins before scraping into tubes. Scraping of the cells was followed by addition of 70% ethanol, mixed thoroughly assuring decreased viscosity and loss of bilayer. All the homogenized lysates were transferred to an RNA binding column, and centrifuged at 12,000rcf for 30 seconds at room temperature. Flow-through was discarded, and low stringency solution was added (from kit, supplemented with 80ml absolute ethanol). Once more, the tubes were centrifuged and flow-through discarded. The tubes were incubated at room temperature for 15 minutes in RNase-free DNase I (reconstituted by 10mM Tris, pH 7.5, and diluted by DNase dilution solution provided 1:15). After incubation, high stringency solution was added for washing (provided by the kit), centrifuged for 30 seconds and flow-through discarded. The cells were washed once more with the low stringency solution, centrifuged for 1 minute and flow-through discarded. To ensure complete removal of wash solutions as ethanol can inhibit down-stream reactions, the tubes were centrifuged for an additional 2 minutes. For elution of the purified RNA, in fresh Eppendorf tubes, elution buffer (supplied by the kit) was pipetted on the membrane stack at the bottom of the RNA binding column, left to saturate for 1 minute, then centrifuged for 2 minutes to recover the isolated RNA.

4.7. Complementary DNA (cDNA) synthesis

Following RNA isolation, the concentrations of the RNA was measured using the NanoDrop 2000 device (Thermo Scientific), using the elution buffer from the kit as a blank. 1µg of total RNA was equalized to 16µL using nuclease free water (Qiagen). Finally, 4µl of the master mix

Bio-Rad (5x iScript Reaction Mix + iScript Reverse transcriptase, iScript cDNA synthesis kit, Bio-Rad) was added in each 16 µL RNA.
The resulting cDNA was 50ng/µl in each tube.

Table 5. c-DNA synthesis program used

Steps	Temperature (°C)	Time (Minutes)
Priming	25	5
Reverse Transcription (RT)	42	30
RT inactivation	85	5

Subsequent successful cDNA synthesis, cDNA was diluted by adding 170µl nuclease-free water, and 10µl of 40x Yellow RNA Loading Dye to make total volume 200µL and concentration of cDNA 5 ng/µL.

4.8. Gene Expression Analysis by Quantitative real-time Polymerase Chain Reaction (qRT-PCR)

100 µM each Gene specific forward and reverse primer stock solutions were diluted in NFW to reach the final concentration of a 5µM final each primer. A mastermix of the primers and SsoAdvanced Universal SYBR green supermix (pre-supplemented with 55 µl of Precision Blue Real-Time PCR dye) was then prepared for each gene, where 2.5µl of SsoAdvanced Universal SYBR green was mixed with 0.5µl of the working solution of the primers previously prepared.

In a 384-well plate, 3µl of the mastermix was pipetted in the each well, then 2µl (10 ng) of the cDNA pipetted in triplicates pattern in each corresponding well. And run according to the given program (Table 6).
Housekeeping genes were GAPDH, RPLP0, RPL13A, TBP, ACTB, HPRT1, B2M and YWAH1
The sequence of the primers of the genes used in qRT-PCR were reported in this study (Castell, Yan, Fawkner, Hydbring, et al., 2018a; Sheng et al., 2018).

Table 6. Quantitative Real-Time Polymerase Chain Reaction program

Steps	Temperature (°C)	Time (Minutes)	
1 Initial Denaturation	95	2:00	-
2 Final Denaturation	95	0:10	-
3 Annealing	6	0:45	
4	-	-	Plateread
5	-	-	Steps 2-4 repeated 40x
6 Melting curve	65	0:31	
7	65	0:05	+ 0,5°C/cycle Ramp 0,5°C C/s
8	-		Plateread
9	-		Steps 7-8 repeated 60x

4.9. Western Blot Analysis

4.9.1. Cells harvesting and Protein isolation

Cells were seeded in 6 cm dishes and harvested and lysed using 200 µL RIPA buffer (supplemented with protease inhibitor cocktail, cOmplete ref: 11697498001, Roche, and phosphatase inhibitor, PhosSTOP, Ref: 04906837001, Roche). The cells were scrapped in RIPA buffer on ice and collected in pre-chilled 1.5 mL eppendorf tubes. Lysates were incubated on ice for 30 minutes, while briefly and gently vortexing every 10 minutes. Following the 30-minute incubation time, samples were centrifuged at 13,200 rpm for 15 minutes at 4°C. In pre-chilled eppendorf tubes, the supernatant was collected and stored at -80°C for further analysis

4.9.2. Protein quantification

The protein quantification was carried out using the Pierce BCA Protein Assay Kit (Thermo Scientific, Ref: 23225).), according to standard procedure. Bovine Serum albumin (BSA, ThermoScientific, 2mg/mL) was used to plot standard curve. The BSA concentrations was 0.5, 1, 1.5, 2, 2.5, 3, 5 and 7 µg/µL used in duplicate in 96 well plate. While 1µl of each sample per well was used. Following a 30-minute incubation at 37°C, the absorbance at 568 nm was

measured using Victor X3 multilabel plate reader (PerkinElmer). The results of the protein quantification were then analyzed and plotted against a linear standard curve of the albumin standard using Microsoft Excel 2007.

Equal volume of protein samples was prepared by addition of 6x Laemmli buffer (provided by Dr. Madhurendra Singh) and heated at 95 C for 5 minutes.

4.9.3. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

A sodium dodecyl sulfate polyacrylamide separating gel and stacking gel were prepared (Table 7) and assembled in the electrophoresis apparatus. The samples and Pageruler Prestained protein ladder (Thermo Scientific) were loaded and ran at 100V for approximately 2 hours.

Table 7. Chemicals used for preparation of the SDS-PAGE Gel

Components	Volume for Resolving gel (12.5 %, 1x)	Volume for Resolving gel (7.5 %, 1x)	Volume for Stacking gel (1x)
Acrylamid/Bis (30%)	2.5 mL	1 mL	0.3 mL
Resolving gel buffer (pH 8,8)	3 mL	3 mL	-
Stacking gel buffer (pH 6,8)	-	-	1.5 mL
dH2O	0.5 mL	2 mL	1.25 mL
N,N,N',N'-Tetramethyl-ethylenediamine (TEMED)	15 µL	15 µL	10 µL
APS (10 %)	45 µL	45 µL	20 µL

4.9.4. Wet Transfer

Following electrophoresis, the gel is wet transferred onto a 0.45 micron nitrocellulose membrane (GE Healthcare Life Sciences). A typical wet transfer sandwich was constructed consisting of the following in order; one foam, 2 Whatman filter papers, the gel, a nitrocellulose membrane, 2 Whatman filter paper, finalized by another foam. The transfer cassette and the nitrocellulose membrane, and all the sandwich-components were pre-soaked in 1x Transfer buffer (1x Tris-glycine transfer buffer, 20% absolute Ethanol and distilled water). After

assembly of the transfer cassette and the ice-block, the chamber was filled to the fullest with 1x transfer buffer, ran at 30V overnight.

On the next day, the membrane was washed thrice with 1X PBS-T (1x PBS, and 0,1% Tween-20). The membrane was stained by Ponceau S solution to rapidly detect transfer of the proteins, before blocking with 5% non-fat dried milk (PanReac AppliChem) in 1X PBS-T at room temperature for 1 hour. The milk blocking solution was used for all antibodies except for the phoshoSTAT1, where it was blocked in 5% bovine serum albumin (BSA, Sigma-Aldrich) in 1x PBST. After blocking the membrane was incubated with the primary antibody (shown in Table 8) in blocking solution overnight at 4C cold-room.

On the following day, the membrane was washed, thrice with 1x PBS-T, and incubated for 2 hours at room temperature in the corresponding HRP-coupled secondary antibody (diluted in 5% milk). Subsequent the incubation in the secondary antibody, the membrane was developed using the SuperSignal West Dura Luminol/Enhancer solution and the SuperSignal West Dura Stable Peroxide buffer (Thermo Scientific) mixed 1:1, in the Bio-Rad Imager with Gel-Doc software.

Table 8. Antibodies used in Western Blot analysis

Antibody	Origin	Company	Dilutions
c-Myc (Y69)	Rabbit	Abcam	1:500
N-Myc (SC-53993)	Mouse	Santa Cruz biotechnology	1:500
P53 (DO-1, SC-126)	Mouse	Santa Cruz biotechnology	1:1000
P21 (Cip1, 610234)	Mouse	BD biosciences	1:500
ISG-15 (SC-166755)	Mouse	Santa Cruz biotechnology	1:500
DNMT1 (ab13537)	Mouse	Abcam	1:500
pSTAT1 (Y701, 9167)	Rabbit	Cell Signalling Technology	1:500
STAT1 (9172T)	Rabbit	Cell Signalling Technology	1:500
β-Actin	Mouse	Millipore	1:10,000

Anti-mouse HRP coupled secondary antibody	Goat	Jackson	1:5000
Anti-rabbit HRP coupled secondary antibody	Goat	Jackson	1:5000

4.10. Flow Cytometry analysis

To investigate the interferon signaling after compounds treatment, the cell lines were transduced with ISRE-mCherry lentivirus (supplied by Dr. Madhurendra Singh). The transduced cells were seeded in 6 cm dishes and treated with MI-6, Nutlin-3 and 7041 using the same conditions mentioned in tables 3 and 4. After 18 hours, the cells were washed, trypsinized and collected in 5 mL FACS tubes. The mCherry intensity was measured using FACS Calibur Flow Cytometer (BD Biosciences) and analysis were done using BD CellQuest software.

4.11. Generation of p53-deficient IMR-32 cells using CRISPR-Cpf1

The cell lines were prepared by Dr. Madhurendra Singh. The generation of p53 knockouts was performed using Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated (CRISP/Cas 12a, also known as AsCpf1) (Bin Moon et al., 2018; Gao et al., 2016).

5. Results

5.1. N-Myc inhibition and p53-reactivation by co-treatment have additive effects on neuroblastoma growth suppression

We wanted to determine the LD50 (Lethal Dose 50%)2 where the cells are 50% dead when we treat with the molecules. To achieve this we used resazurin viability assay using two-fold serial dilutions of the compounds in neuroblastoma cell lines (p53 WT; N-Myc non-amplified – SHSY-5Y, p53 WT; N-Myc amplified – IMR32, p53 mutant; N-Myc non-amplified – SKNAS while MI-6 showed reduced growth suppression only in N-Myc amplified cell lines at lower concentration. While N-Myc non-amplified cells had growth suppression at mid -high concentration (2.5 uM). The p53 mutant cells showed minimal or no growth suppression by p53-WT reactivating compound **(Fig. 5C and D)**.

In MI-6 and 7041 combination treatment, all cell lines showed either enhance (SHSY-5Y) or like alone single compound treatment **(Fig. 5B-D)** growth suppression. Based on these experiment we have chosen SHSY-5Y and IMR-32 using 1 μM of 7041 and 2 μM of MI-6 for further experiments. and p53 mutant ; N-Myc amplified SK-BE2) **(Fig.5). In p53-WT cells,** SH-SY5Y **(5A)** and IMR32 **(5B)**, alone 7041 treatment have drastic growth suppression at lower concentration,

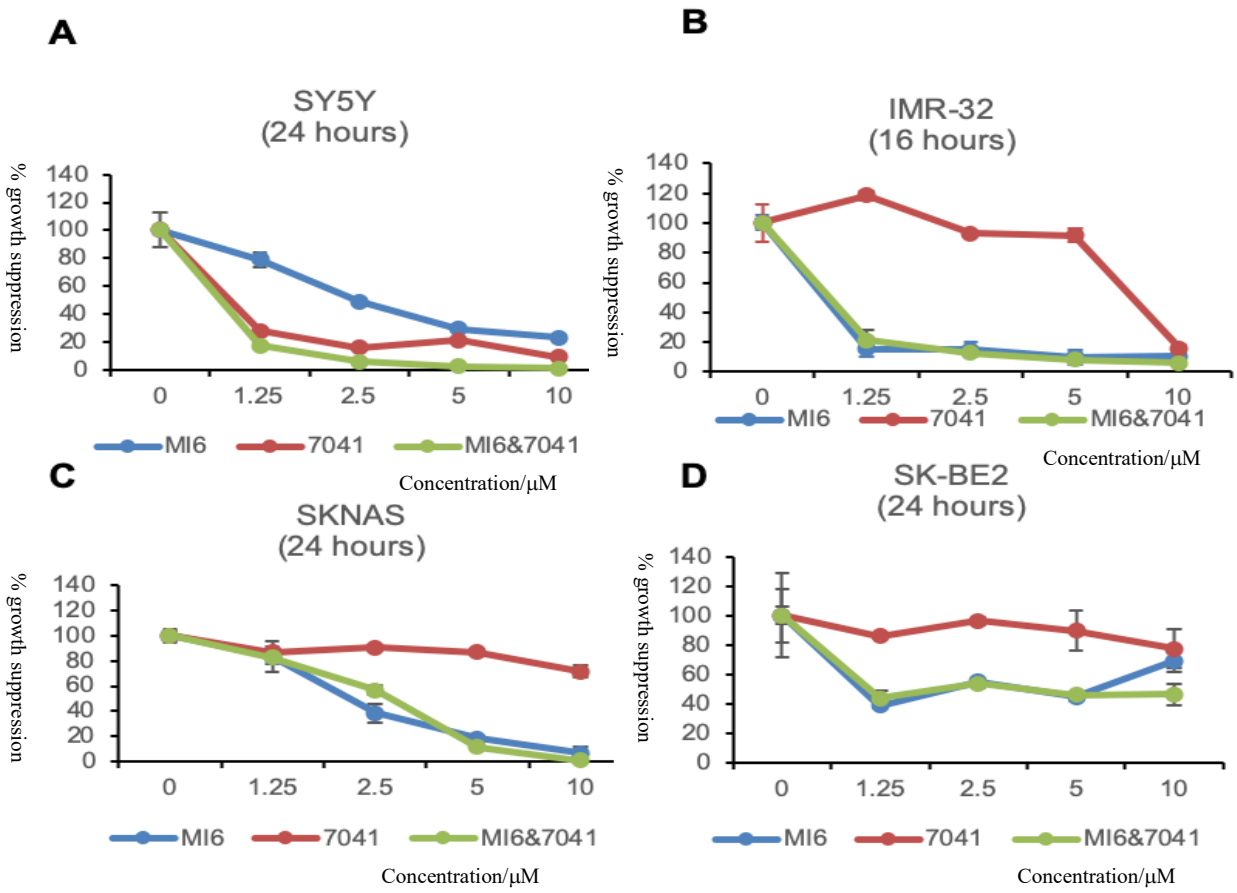


Figure 5: Cell viability using Resazurin assay in four different neuroblastoma cell lines; SH-SY5Y, IMR-32, SK-N-AS and SK-BE2. All the cells were treated with MI-6, 7041 and combination of MI-6&7041, in two-fold serial dilution. The serial dilution was as follows: 10µM, 5µM, 2.5µM, 1.25µM, 0µM (DMSO). **A)** SH-SY5Y cell line, the cells were treated for 24 hours. **B)** IMR-32 cell line, the cells were treated for 16 hours. **C)** SK-N-AS cell line, the cells were treated for 24 hours. **D)** SK-BE2 cell line, the cells were treated for 24 hours. The x-axes in all charts show: concentration of compounds in µM, while the y-axes show % of viable cells (normalized to DMSO treated cells.) The error bars represent the SD.

5.2. Validation of p53 and MYC target genes expression using compounds treatment in neuroblastoma

Next, we verified the compounds activity in cells by monitoring their target-regulated gene expression at mRNA and at protein levels **(Fig. 6)**. To verify MI-6 dependent N-Myc inhibition, we have chosen N-Myc regulated genes - GLUT1, HK2, CAD, TPI1, PFKM, c-Myc and N-Myc (Castell, Yan, Fawkner, Zhang, et al., 2018). And as p53 repress Myc expression, c-MYC and N-MYC were used to verify p53-dependent 7041 or nutlin activity.

All genes were downregulated following treatment; however, Nutlin-3 showed the least efficacy, while MI6&7041 was the most effective treatment condition in almost all genes.

Nevertheless, in IMR-32 **(Fig. 6C)**, treatment was less effective than in other cell lines, which suggests that 8 hours is less effective than 16-hour treatment time point. Western blot analysis for SH-SY5Y **(Fig. 6B)** and IMR-32 **(Fig. 6D)** cell lines was then performed to examine the protein levels of the N-Myc, c-Myc, p53 and p21, to validate the qPCR results. The same treatment conditions with the same concentrations were followed.

In SH-SY5Y, N-Myc protein level showed extreme downregulation in MI-6 treatment, and slight down regulation in MI-6&7041 and MI6&Nutlin-3 treatment. In addition, c-Myc was down regulated slightly after treatment of MI-6&7041. While p53 and p21 protein levels were highly upregulated after treatment with 7041 and combination, and slightly upregulated after treatment with Nutlin-3 and MI-6&Nutlin-3.

In IMR-32 cell line, c-Myc protein level was slightly downregulated following treatment, N-Myc was marginally downregulated subsequent MI-6&Nutlin treatment, while p53 and p21 were remarkably upregulated after treatment with 7041 and MI6&7041. On the contrary, p53 and p21 protein levels were upregulated but with less intensity under Nutin-3 and Nutlin-3&MI6 treatment conditions, in comparison to 7041.

Concluding that, the compounds under study are functioning adequately. Therefore, we proceeded with investigating the effect of p53 and N-Myc on the tumor immunogenicity.

5.3. Antigen processing and presentation genes upregulation after p53 activation and Myc inhibition in SY5Y and IMR32 cells.

The antigen and processing cells will express MHC class I loaded with antigen to get recognize by T cells. It is a process where protein antigen is endocytosed by an antigen-presenting cell (APC), broken down into peptide fragments and presented on the surface of the APC with an antigen-presenting molecule such as MHC class I or MHC class II. Some of those antigen processing and presenting genes include β2M, ERAP1, ERAP2, TAP1, TAP2, MHC class I (HLA-A, HLA-B, HLA-C) (Mak et al., 2014). Also as previously mentioned that, the host T-cell compartment evidently turn a blind eye to neuroblastoma cells due to the limited expression of HLA and costimulatory molecules on antigen presenting cells, which as we discussed are consequential for effective peptide presentation to T cells (Prigione et al., 2004).

Therefore, the genes involved in antigen processing and presentation were examined using qPCR in SH-SY5Y **(Fig.7)** and IMR-32 **(Fig.8)**. After treatment of the cells with MI-6, 7041, and combination, in SH-SY5Y, HLA-B (1.8 fold), HLA-C (1.8-fold), β2M (1.6-fold), TAP1

(7-fold), TAPBP1 (1.2-fold) and ERAP2 (1.8-fold) were upregulated in comparison to DMSO-treated cells. Additionally, in IMR-32 p53 wild-type cells, these genes HLA-B (1.4-fold), HLA-C (1.7-fold), β2M (1.7-fold), TAP1 (2-fold), TAPBP1 (1.9-fold), ERAP1 (1.8-fold) and ERAP2 (1.6-fold) were upregulated when compared to DMSO-treated cells under the same conditions. In IMR-32, all the antigen processing presentation genes were p53 dependent, as the upregulation of most genes were absent in p53-KO cells after treatments.

5.4. Induction of anti-viral Type I Interferon beta after p53 activation and N-Myc inhibition

Since tumor cells behave similarly to virus-infected cells, they radiate danger signals to warrant immune system, type-I interferons have a lead role for the following events linking innate and cognate immunity (Sistigu et al., 2014). Furthermore, it has been proposed that the MHC-class I processing and presentation genes are under control of JAK-STAT signaling (Goel et al., 2017). Also, type-I-IFNs forms a bond with a heterodimeric transmembrane receptor called IFN-α/β receptor (IFNAR) which activates JAK-STAT signaling. As a result, the transcription of numerous IFN-stimulated genes' (ISGs) is triggered. On the grounds of these findings, the levels of type-I interferon were a point of interest in our experiment.

Therefore, first we have established Interferon-reporter assay using 8x ISRE-mCherry in IMR-32 and SHSY-5Y cells **(Fig.9)**, the ISRE Reporter is designed for monitoring the activity of Type I interferon-induced JAK/STAT signaling pathway in the cultured cells.
In both cell lines, single treatment of 7041, Nutlin or MI-6 have minimal activation of interferon signaling, while combination of p53-activation and Myc-inhibition have significantly increased mCherry expression as monitored by flow cytometry.
To further validate this finding, we checked the gene expression levels of Interferons and Interferon-regulated genes. Interestingly, Type I INF-β was significantly induced **(Fig. 10A)** after combination. Moreover, the INF-β upstream transcription factor IRF7 was also significantly induced. Which suggest that Interferon signaling is induced after combination treatment. To strengthen our finding, we also performed INF-β regulated genes IFN-28A and ISG56. We found those were significantly induced after N-Myc inhibition and p53 activation. We did not observe changes in other genes it is might because of technical issue, due to time limitation I was unable to perform more experiments.

Furthermore, on protein level we could observe induction of phosphoSTAT1 and ISG-15 in IMR-32 cells **(Fig. 10B).** Which confirm our finding that combination treatment has enhance anti-viral innate immune response.

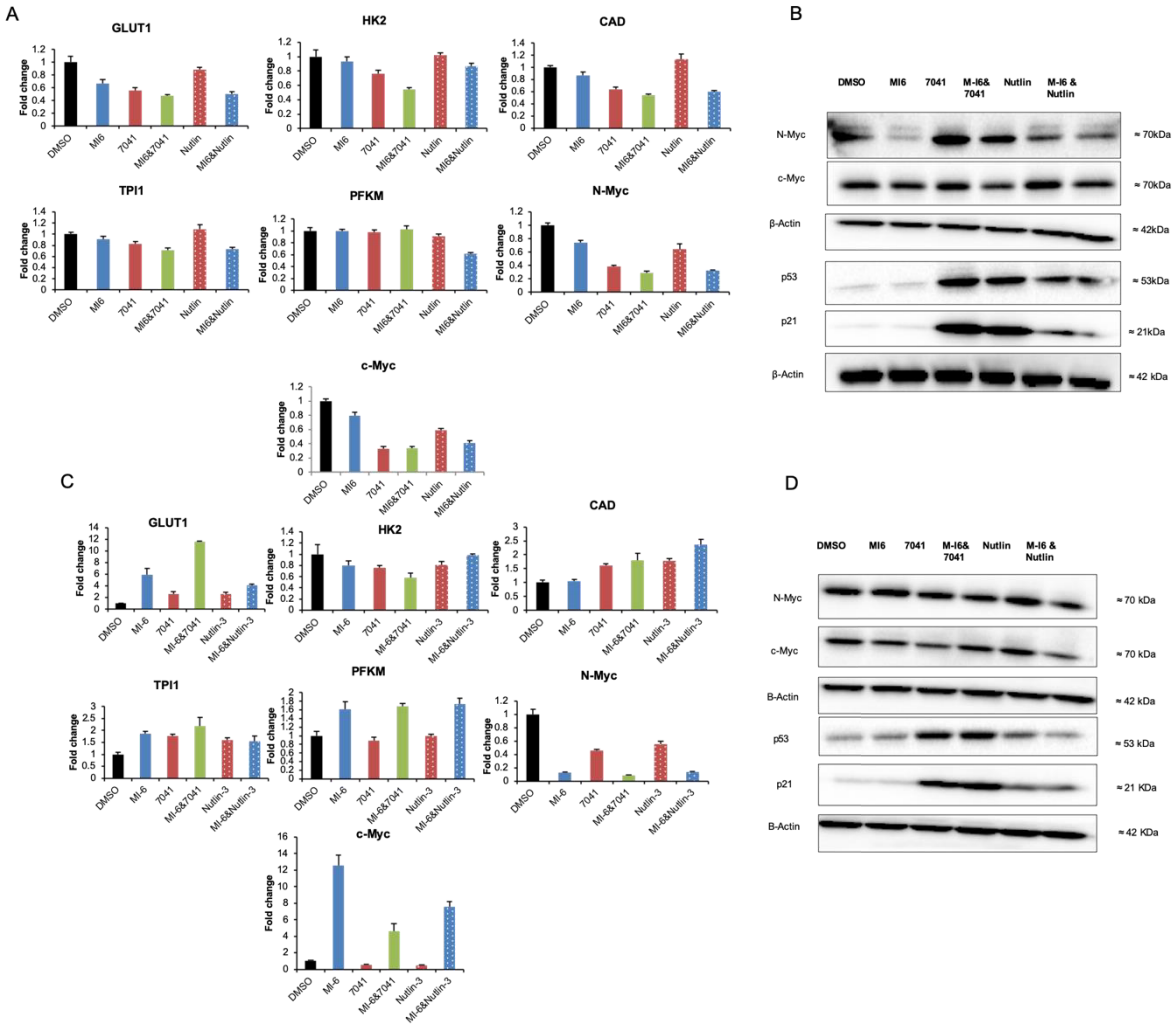


Figure 6: Confirmation of the compounds activity. Each cell lines were seeded in 6-well plates. The same treatment pattern was followed; DMSO (black), MI-6 (2μM, blue), 7041 (1μM, red), MI-6&7041 (green), Nutlin-3 (1μM, red-coloured with pattern fill) and MI-6& Nutlin-3 (blue coloured with pattern fill). **A)** SH-SY5Y cell line qPCR analysis showing the RNA expression levels of the genes after treatment for 16h. y-axes show fold change **B)** Western blot analysis for SH-SY5Y showing N-Myc (70 kDa), c-Myc (70 kDa), p53 (53 kDa), and p21 (21 kDa) protein levels after treatment. β-actin (42 kDa) was used as a loading control. **C)** IMR-32 cell line qPCR analysis showing the RNA expression levels of the control genes. The cells were treated for 8 hours. Y-axes show fold change **D)** Western blot analysis for IMR-32 showing protein levels of N-Myc, c-Myc, p53 and p21. β-actin was used as a loading control. All experiments were performed n=1

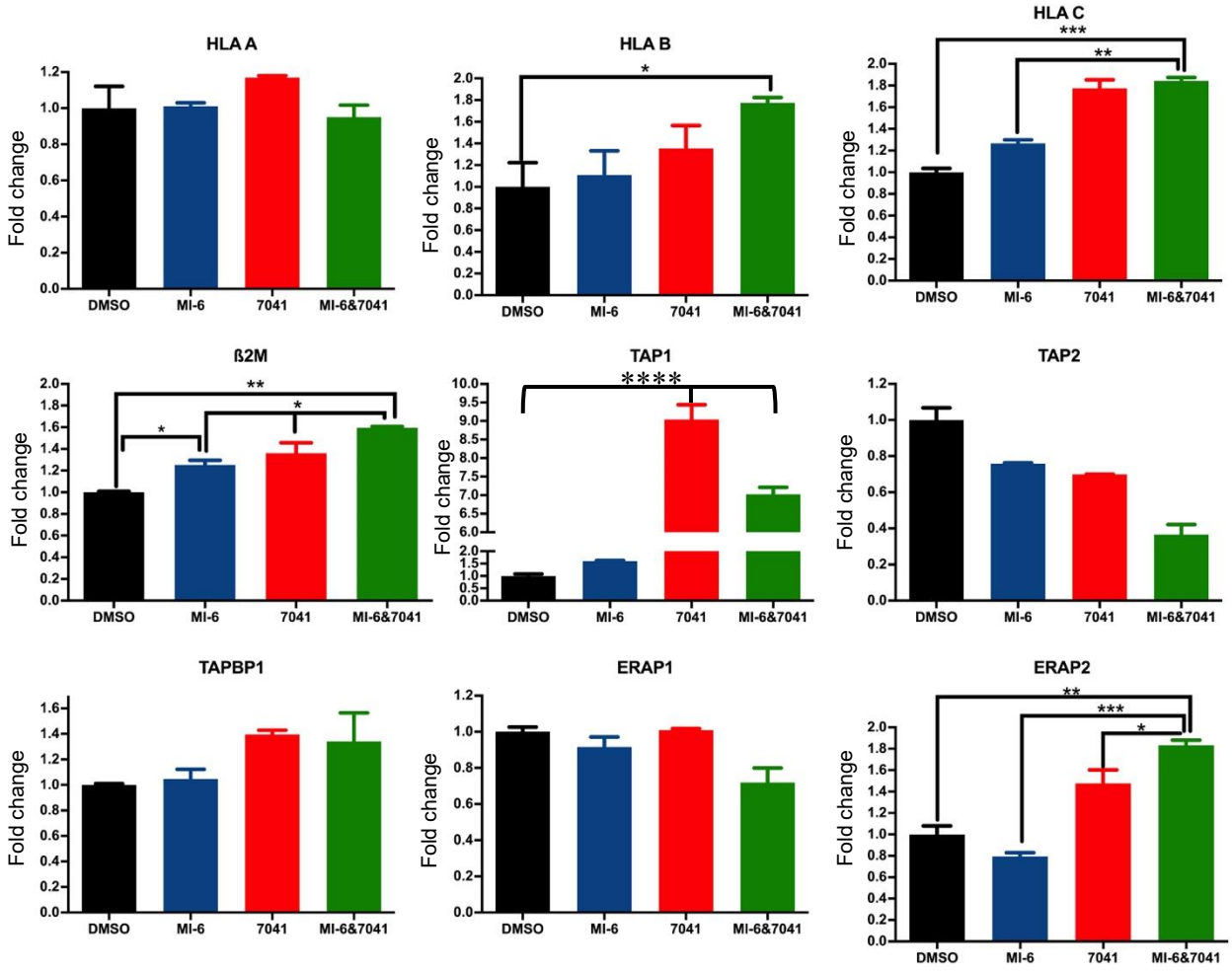


Figure 7: Antigen processing and presentation genes upregulation after p53 activation and Myc inhibition in SH-SHSY-5Y. qPCR analysis was performed to check the RNA expression levels of the MHC class genes. Treatment conditions were DMSO, MI-6 (2μM, blue-coloured), 7041 (1μM, red-coloured) and MI-6 and 7041 (green-coloured) for 16 hours. Each bar represents the mean with the standard error of mean (SEM) of at least two experiments. P-values were calculated by one-way ANOVA test and differences in gene regulation between treatment were compared using post-hoc Fisher's LSD test. (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001). The real-time qPCR presented as fold changes of gene expression compared to DMSO.

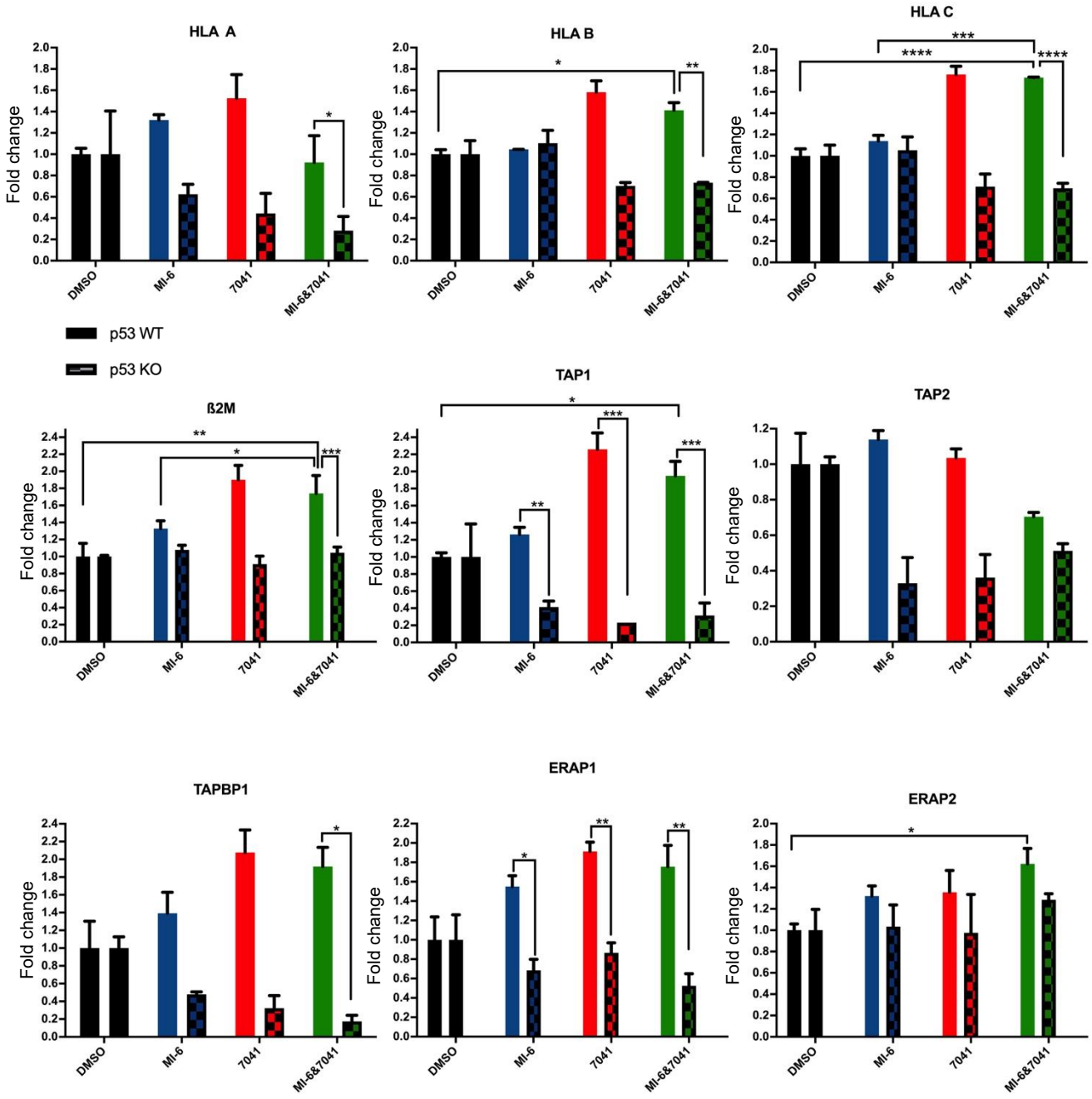


Figure 8: p53-depepndent antigen processing and presentation genes upregulation in IMR-32 cells. qPCR analysis was performed to check the RNA expression levels of the MHC class genes. Treatment conditions were DMSO, MI-6 (2μM, blue-coloured), 7041 (1μM, red-coloured) and MI-6 and 7041 (green-coloured) for 16 hours. The p53 wild-type cells are solid-filled while the p53 knockouts are pattern filled.
Each bar represents the mean with the standard error of mean (SEM) of at least two independents experiments. P-values were calculated by one-way ANOVA test and differences in gene regulation between treatment were compared using post-hoc Fisher's LSD test. (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001). The data presented as fold changes of gene expression compared to DMSO.

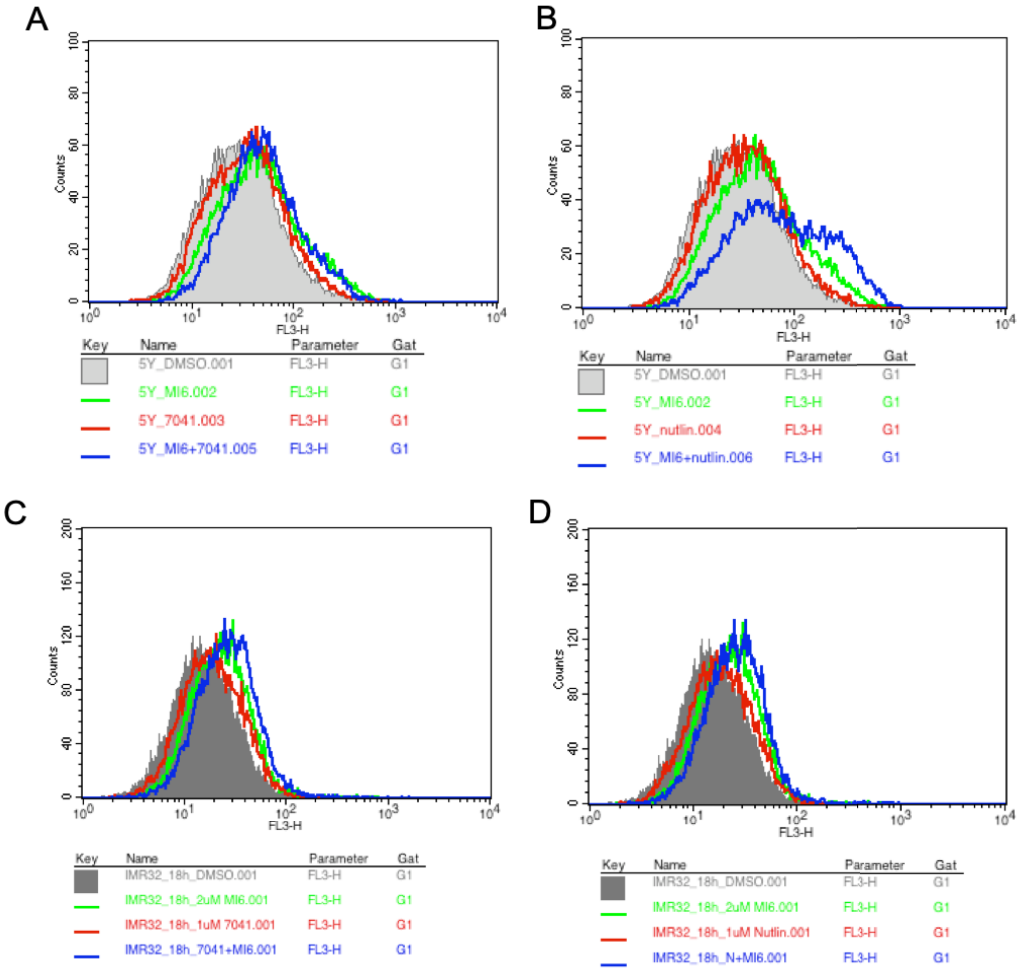
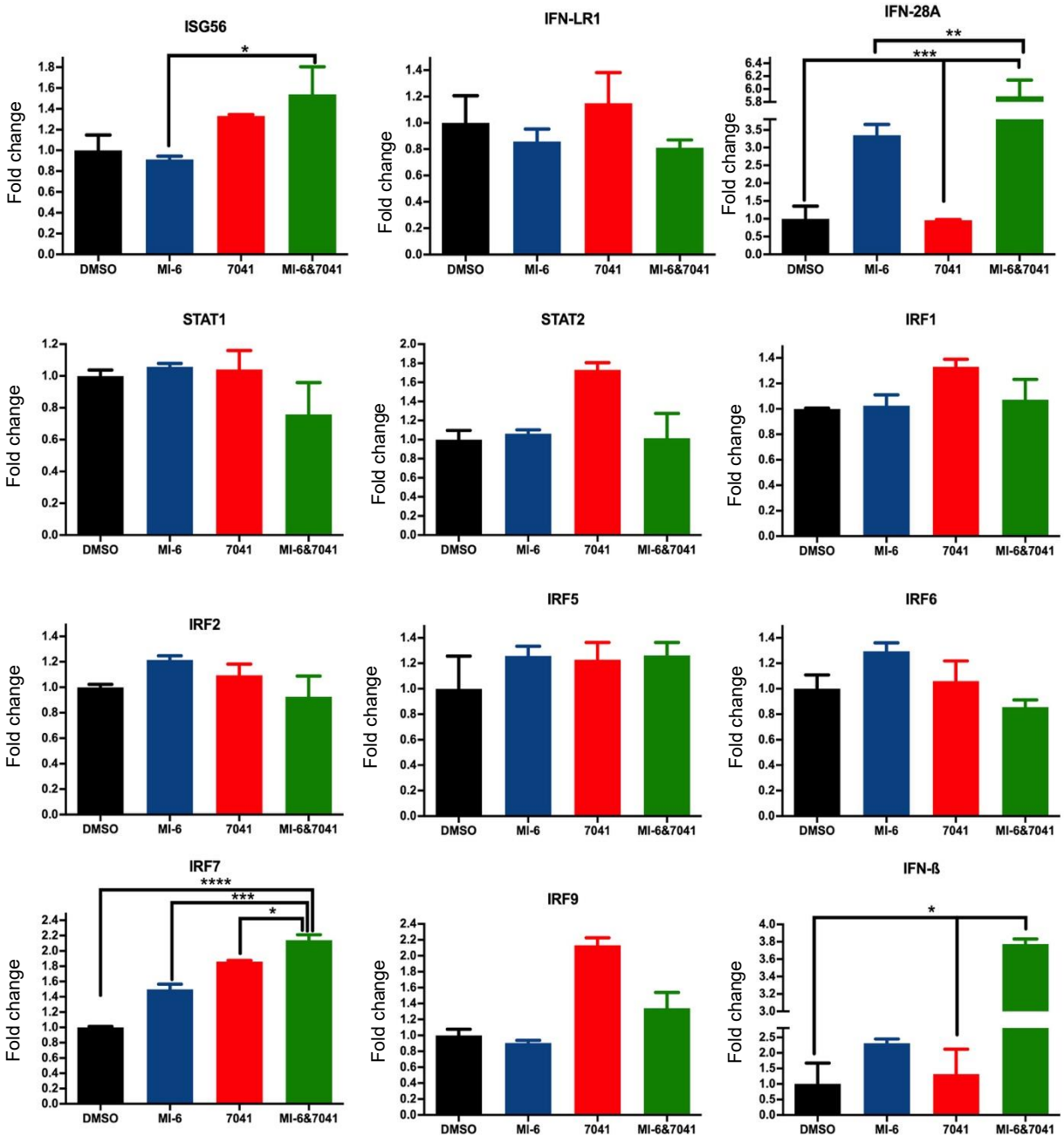


Figure 9: Induction of Interferon signaling after successive treatment with the molecules in SH-SY5Y and IMR-32 neuroblastoma cell lines. A) Interferon signaling in SH-SY5Y after 16hr treatment with DMSO (grey-coloured), MI-6 (2μM, green coloured), 7041(1μM, red coloured) and combination of these two small molecules (blue-coloured). **B)** Interferon signaling in SH-SY5Y after 16hr treatment with DMSO, MI-6 (2μM), Nutlin-3 (1μM, red-coloured) and combination of these two small molecules (blue-coloured). **C)** Interferon signaling in IMR-32 after 18hr treatment with DMSO, MI-6, 7041 and combination of these two molecules. **D)** Interferon signaling in IMR-32 after 18hr treatment with DMSO, MI-6, Nutlin-3 and combination of these two small molecules. Experiments were performed n=2

A



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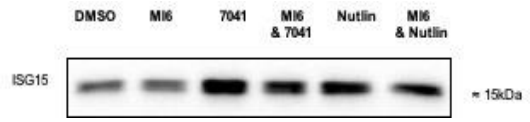

Figure 10: Induction of interferon-signaling after combination treatment in SH-SY5Y neuroblastoma cell line. A) qPCR analysis was performed to check the RNA expression levels of the STAT/IFN pathway genes, the second checkpoint. Treatment conditions were DMSO, MI-6 (2µM, blue-coloured), 7041 (1µM, red-coloured) and MI-6 and 7041 (green-coloured) for 16 hours. Each bar represents the mean with the standard error of mean (SEM) of at least two experiments. P-values were calculated by one-way ANOVA test and differences in gene regulation between treatment were compared using post-hoc Fisher's LSD test. (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001).

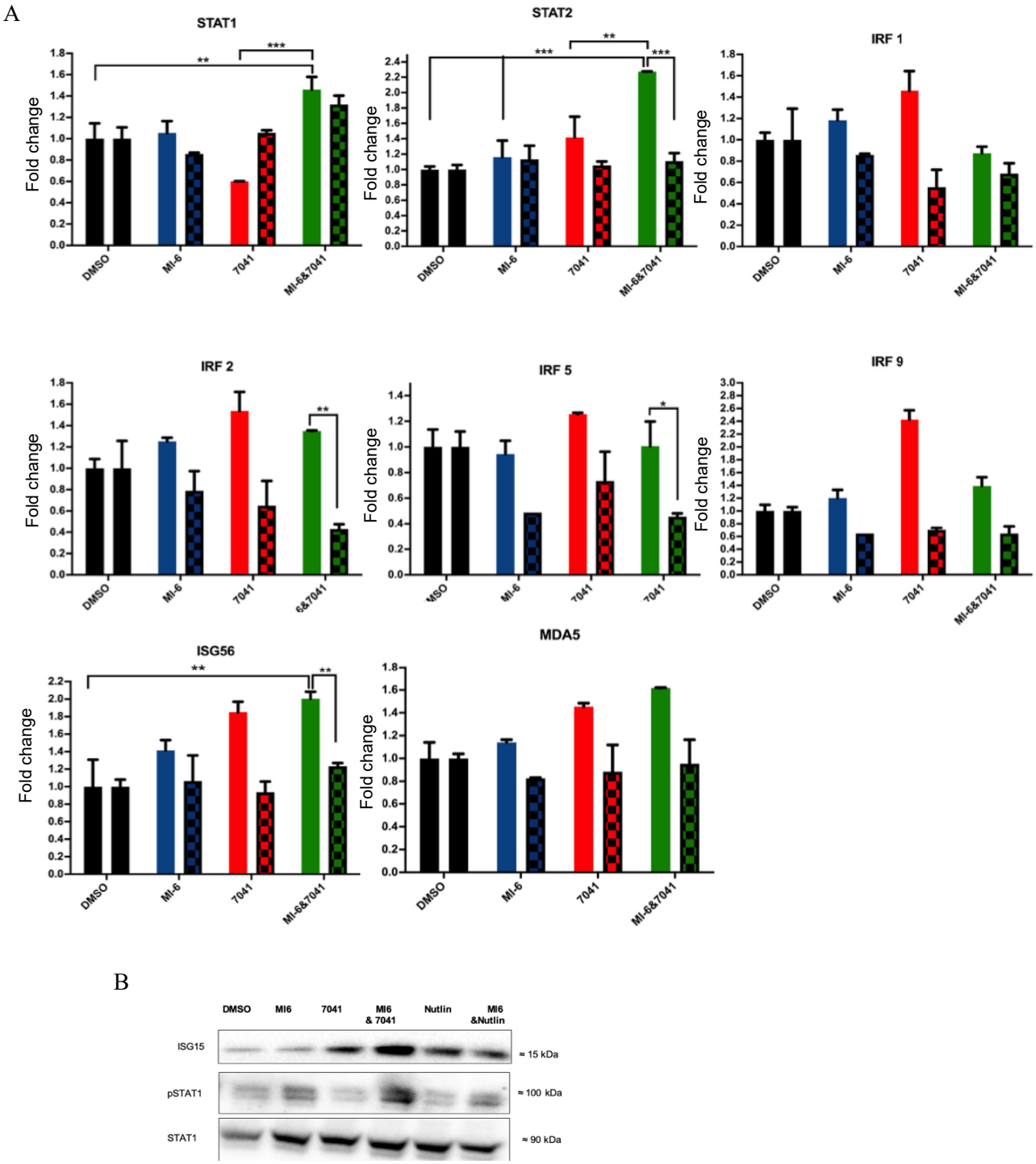

Figure 11: Induction of interferon-signaling after combination treatment in SH-SY5Y neuroblastoma cell line in IMR-32 neuroblastoma cell line. A) qPCR analysis was performed to check the RNA expression levels of the STAT/IFN pathway genes. Treatment conditions were DMSO, MI-6 (2μM, blue-coloured), 7041 (1μM, red-coloured) and MI-6 and 7041 (green-coloured) for 16 hours. The p53 wild-type cells are solid-filled while the p53 knockouts are pattern filled. Each bar represents the mean with the standard error of mean (SEM) of at least two experiments. P-values were calculated by one-way ANOVA test and differences in gene regulation between treatment were compared using post-hoc Fisher's LSD test. (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001). **B)** Protein level of ISG-15 and pSTAT1 after treatments for 18 hours.

5.5. Induction of endogenous retroviruses (ERVs) and dsRNA receptor after combination treatments

The concept of immunoediting as previously mentioned hypothesize that cancer cells has the capability to wind down immunogenicity to circumvent destruction by the host's immune system. Endogenous retroviruses are retroviruses that are present in the human genome (around 8% of the human genome) although it is almost majorly silenced epigenetically including DNA methylation via Dnmt1 in the CpG sites or by deactivating mutations. It has been found that when ERVs are activated or their expression is elevated in human cells, an immune response is initiated through the awakening of innate and adaptive immunity (Gonzalez-Cao et al., 2016).

Previous literature has demonstrated that DNA methyltransferase inhibitors (DNMTi) elevate immune response in tumor via a viral defense channel. DNMTi tend to activate cytosolic sensing of double-stranded RNA (dsRNA) which in turn triggers a Type I interferon response and cell death. When ERVs are upregulated or overly expressed, they further trigger the interferon response as mentioned above (Chiappinelli et al., 2015). It has also been shown that 5-Aza-deoxycytidine (DNMTi) activates p53 signaling, consequently apoptosis is triggered. This proposes that the DNA methylation action exerted by DNMTs is linked to p53 signaling (Y. A. Wang et al., 2005). Another study has validated that p53 and RB genes transcriptionally inhibit DNMT expression (Tang et al., n.d.), thus, ERVs are activated in consequence.

Accordingly, the third checkpoint we wanted to study the effect of our treatment on, after proving successful effectiveness on the first two checkpoints, was the induction of ERVs. qPCR analysis was performed to check the RNA expression levels of different ERVs in SH-SY5Y **(Fig. 12)** and IMR-32 **(Fig. 13)**.

The ERVs tested using qPCR were: ERV-E, ERV-F, ERV-K, ERV-L, ERV-WE1 envelope protein, ERV-WE1, LTR26E, and ERV3 envelope protein. Some of these were not expressed in our model cell lines, and others were not significantly changed.

The gene expression levels of SH-SY5Y showed induction in ERV K, ERV E, ERV L, ERV WE1, ERV WE1 envelope protein, and in long terminal repeat 26E (LTR26E). Furthermore. IMR-32 cells showed upregulation in ERV E, ERV K and ERV3 envelope protein. In this cell line, the dependency on p53 activation was proven, as the p53 knocked-down cells showed contrast results to the p53 wild-type cells. This data suggests the advantageous and successful

activation of the third checkpoint of the pathway, by the reason of induction of RNA expression levels of ERVs following treatment with MI-6 and 7041. . The ERVs can transcribe bi-directionally by both sense and anti-sense strand of DNA and thus form dsRNA in the cytoplasm. The ERVs in cytoplasm can be recognized by the dsRNA sensors: TLR3, MDA5 and RIG-1 and transduce the signal to MAVS (Chiappinelli et al., 2015). Moreover, we could see significant induction of TLR3, MAVS and RIG-1 after combination treatment. These results confirm the induction of ERVs dictate type I interferon signaling.

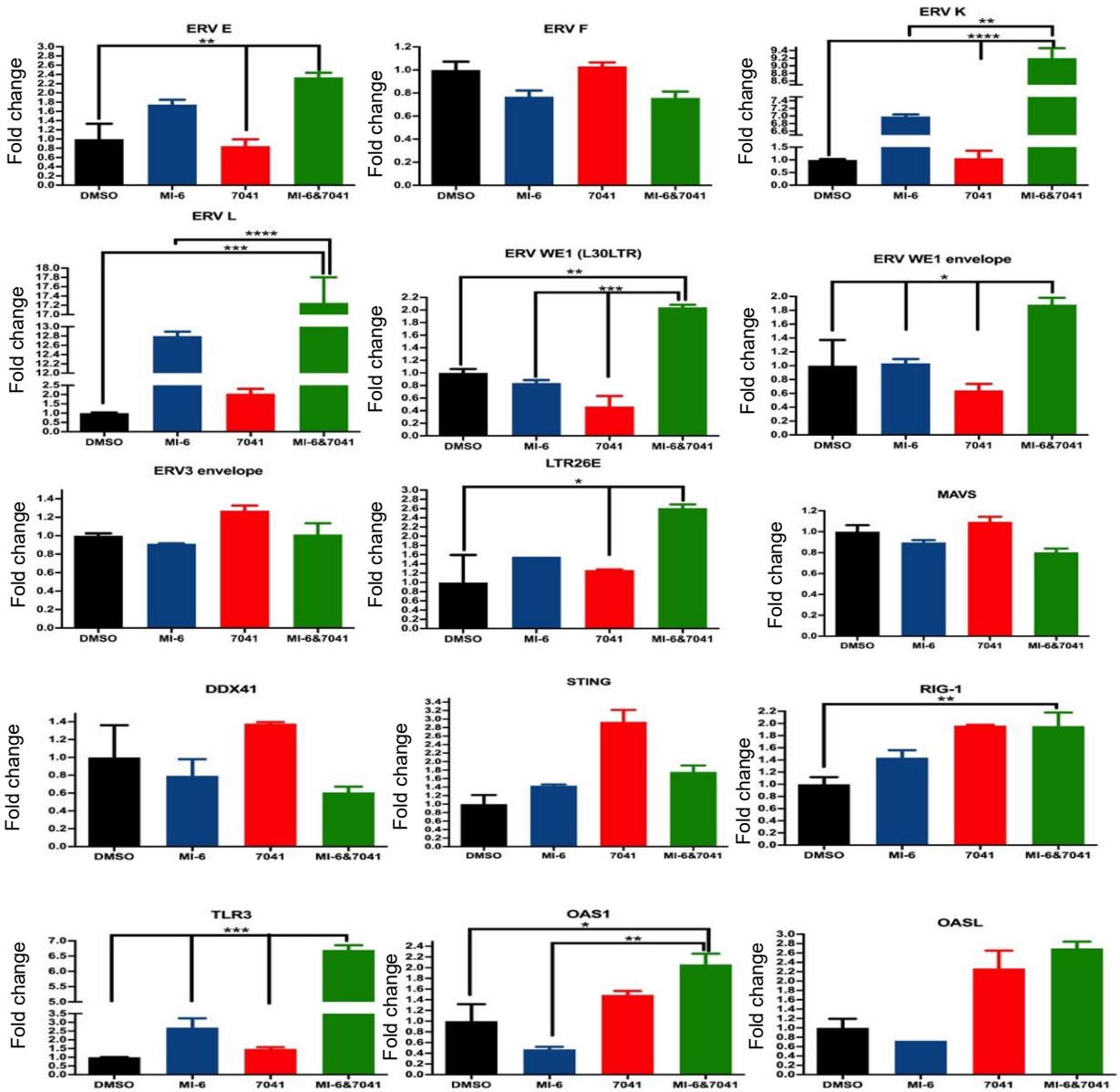

Figure 12: Induction of endogenous retroviruses (ERVs) and dsRNA receptor after combination treatment in SH-SY5Y. qPCR analysis was performed to test the effect of treatment on the RNA expression levels of ERVs. Treatment conditions were DMSO, MI-6 (2µM, blue-coloured), 7041 (1µM, red-coloured) and MI-6 and 7041 (green-coloured) for 16 hours. Each bar represents the mean with the standard error of mean (SEM) of at least two independents experiments. P-values were calculated by one-way ANOVA test and differences in gene regulation between treatment were compared using post-hoc Fisher's LSD test. (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001).

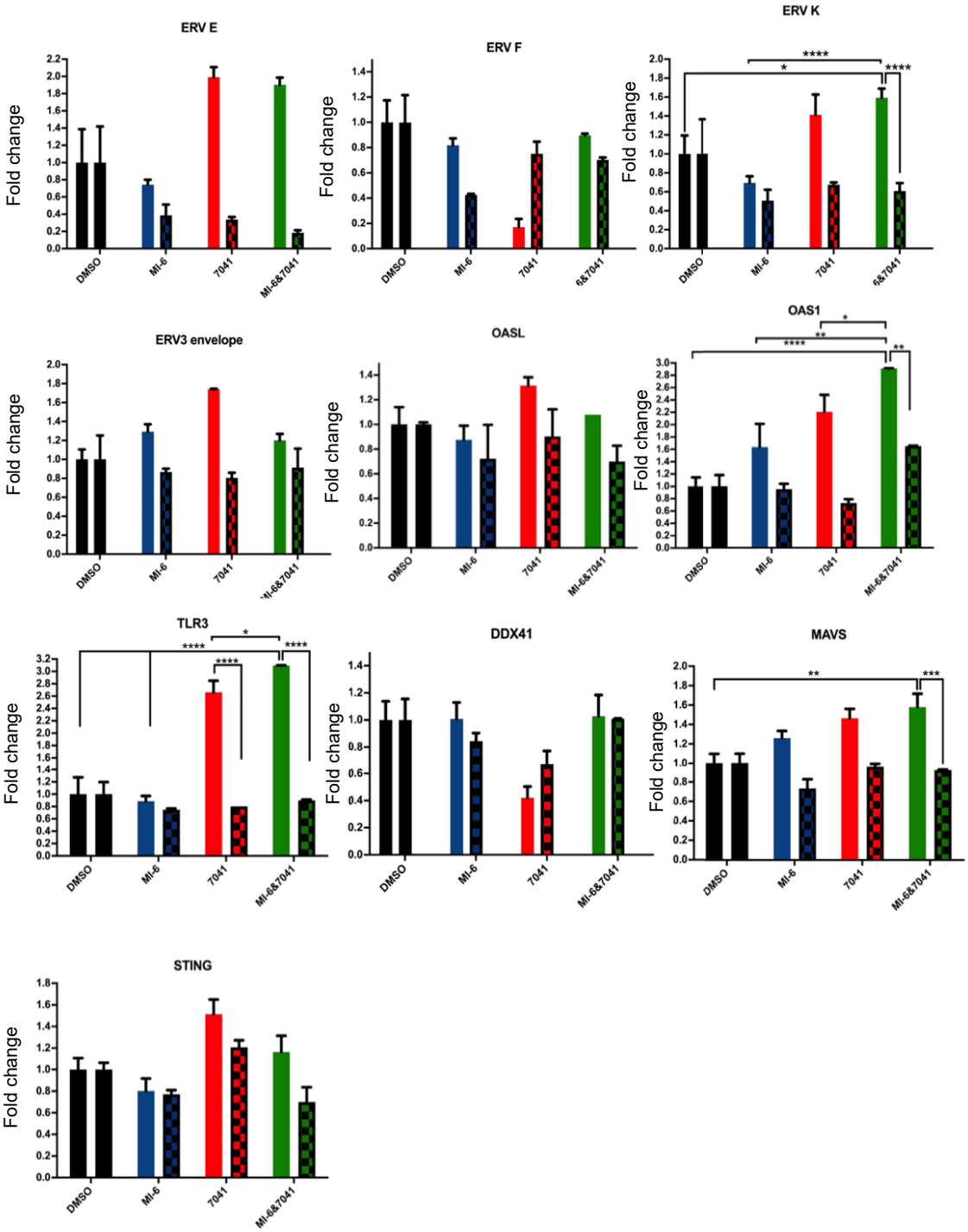

Figure 13: p53-dependent induction of endogenous retroviruses (ERVs) and dsRNA receptor after combination treatment in IMR-32. qPCR analysis was carried-out to test the effect of treatment on the RNA expression levels of ERVs. Treatment conditions were DMSO, MI-6 (2μM, blue-coloured), 7041 (1μM, red-coloured) and MI-6 and 7041 (green-coloured) for 16 hours. The p53 wild-type cells are solid-filled while the p53 knockouts are pattern filled. Each bar represents the mean with the standard error of mean (SEM) of at least two experiments. P-values were calculated by one-way ANOVA test and differences in gene regulation between treatment were compared using post-hoc Fisher's LSD test. (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.

5.6. DNMT-1 repression after combination treatment responsible for observed ERVs expression

As mentioned before, the ERVs promoter are highly methylated and their expression is silenced (Chiappinelli et al., 2015). We hypothesized that the upregulation of ERVs is might due to repression of DNMT1 by inhibition of MYC and activation of p53.

To validate this hypothesis, we checked the mRNA and protein expression of DNMT1 after compounds treatment. Indeed, the mRNA expression of DNMT1 (**Fig. 14A and C**) in both cell lines were significantly reduced after combination treatment. Moreover, at DNMT1 protein level, combination showed more reduction (**Fig. 14B and D**).

Taken together, our result suggest the p53 activation and Myc repression in neuroblastoma leads to repression of DNMT1 at ERVs promoter which leads to expression and accumulation of dsRNA stress and Type I interferon signaling mediated induction of tumor immunogenicity (**Figure 15**).

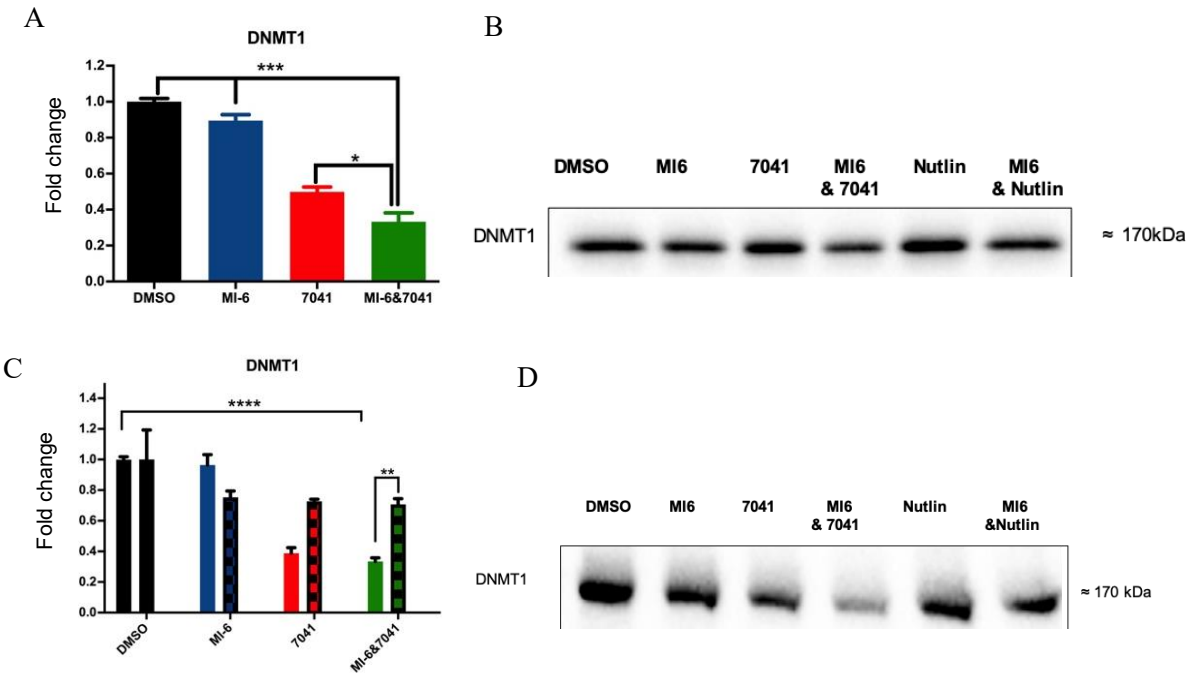

Figure 14: DNMT1 down regulation after combination treatment. A) qPCR analysis was performed to check the RNA expression levels of DNMT-1 after treatment in SY5Y **B)** Western blot analysis for SH-SY5Y showing protein levels of DNMT-1 following treatment with MI-6 and 7041. β-actin was used as a loading control (Shown in fig. 5B). **C)** qPCR analysis was performed to check the RNA expression levels of DNMT-1 after treatment in IMR-32 **D)** Western blot analysis for IMR-32 showing protein levels of DNMT-1 following treatment with MI-6 and 7041. The same treatment conditions were followed in both qPCR and western blot analysis. Experiment n=2

6. Discussion

To eliminate cancer, we need to fully activate the host immune system, which has the capacity to distinguish self and non-self-antigens. The tumor p53 have shown to function as an important activator of innate immunity (Sanz et al., 2019). In contrast oncogene Myc have opposing function in both innate and adaptive immunity. In this project, we try to address important question: does p53-activation and Myc-inhibition in neuroblastoma leads to increased tumor immunogenicity?

Our findings suggests, 1) combination of MYC inhibitor and p53-activators lead to increased expression of antigen processing and presentation genes 2) the enhanced activation of antigen processing and presentation genes is due to increased anti-viral type I interferon beta signaling. 3) the expression and production of INF-β is under control of ERVs derived dsRNA production and their binding to dsRNA sensors in the cells 4) the inhibition of DNA (cytosine-5)-methyltransferase 1 is an enzyme, DNMT1 after combination might be responsible for observed enhanced expression of ERVs and down-stream signaling cascade.

TP53 is a gene that codes for a p53 protein that governs numerous pathways such as metabolic reprogramming, autophagy, cell cycle arrest and apoptosis. It is a master regulator for cells and has pivotal role in multicellular organisms to suppress cancer; however, it is often found mutated or inactivated. Furthermore, through previous literature showing an intact p53 pathway, it has been recently shown or evidenced that p53 inactivation might be the remarkable mechanism of drug resistance found in neuroblastoma cell lines to date. The study of p53 in neuroblastoma has emerged some observations of p53 accumulation and probable inactivation due to cytoplasmic sequestration (Tweddle et al., 2003). Another stapled α−helical peptide has emerged, ATSP-7041 (Aileron therapeutics USA, clinical trial phase I). ATSP-7041 is a potent dual inhibitor of MDM2 and MDMX which consequently activates p53 pathway in tumors (Chang et al., 2013a). Another p53 inducing small molecule, which inhibits p53-MDM2 interaction, Nutlin-3 (Roche, nutlin-3 like molecule in clinical trial phase III) (Vassilev et al., 2004), was used in this study.

Myc also has a pivotal role in cancer, and in the maintenance of the cancer cells. It augments the genesis of many human cancers as it is a pleiotropic transcription factor that is existent at the crossroads of multiple pathways that is key of the cells' fate. It can solely drive cancer progression and tumor growth (Castell, Yan, Fawkner, Zhang, et al., 2018).

Therefore, targeting activation of p53 and/or inhibition of MYC could be a potential therapy to induce immunogenicity in the fatal neuroblastoma. Previous research found a small molecule MYCMI-6 which blocks MYC-driven transcription and binds selectively as explained before, to the MYC bHLHZip domain (Castell, Yan, Fawkner, Zhang, et al., 2018). In this study, they originally experimented several MYC inhibitors, however, MYCMI-6 was the one of the specific and potent MYC:MAX inhibitor.

It has been previously observed that one reason that contributes to the neuroblastoma metastatic trait is the amplification of N-Myc gene. When N-Myc is amplified, MHC class I is repressed, however, this action could be remodeled again following interferon induction (Bernards et al., 1986b). p53 has demonstrated strong direct transcriptional influence on IFN-inducible genes as IRF9, IRF5, ISG15 and TLR3. This exhibits how p53 has a central lead in activation of antiviral innate immunity either through apoptosis or through encouraging type I IFN response (Rivas et al., 2010). Furthermore, a previous study showed that MYC can negatively impact STAT1 (A primary transcription factor stimulated by interferons and has a central role connecting the Type I and Type II interferon responses). Thus, inhibition of MYC expression results directly or indirectly in stimulation of STAT1, this in turn activates and triggers Type I interferons (Schlee et al., 2007). Also, IRF7 which is a crucial governor of Type I IFN response was found to be depleted or greatly repressed by MYC. It was shown that MYC is found at the IRF promoter region together with a nuclear receptor corepressor 2/histone deacetylase 3. When MYC was inhibited, IRF7 expression was greatly induced (T. W. Kim et al., 2016). From these findings, we wanted to test the effect of the combined treatment (N-Myc inhibition and p53 activation). It was also found that ERVs can function as tumor-associated antigens activating both T-cell and B-cell immune responses in tumors (Wang-Johanning et al., 2008). Nevertheless, whether N-myc inhibition and/or p53 activation treatment would have the same effect in neuroblastoma cell lines or the possible effect on the tumor immunogenicity has not been covered yet. In our study this is what we aimed for, after those collective findings, we were intrigued to investigate how and if the induction of immunogenicity in the fatal neuroblastoma could be possibly induced using co-treatment.

Following combination treatment, we observed remarkable interferon signaling, and induced IFN-β RNA levels. Consequently, activation in some of the antigen processing and presenting genes was noted in both cell lines using qPCR. And on protein level, ISG-15 and pSTAT1 were also induced. Our results suggest significant induction in comparison to single-treatment,

as well as p53-dependency in IMR-32 cells causing this noted induction (as demonstrated in the results section).

Myc was found to suppress transcription by functional involvement with transcriptional activating genes. Myc through the interaction and linking with Dnmt3a and DNMTs activity *in vivo*, represses the p21Cip1 (implied in p53 mediated inhibition of cell proliferation) gene promoter (Brenner et al., 2005). This shows the intertwined role of Myc in encouraging cell proliferation and growth even further by interfering with p53. While, it was shown that when p53 is overexpressed, DNMT1 protein expression is repressed (L. Zhang et al., 2016). Correspondingly, our combination treatment in neuroblastoma by inhibiting Myc and activating p53 couple between these findings, possibly exhibiting stronger impact on the overall tumor immunogenicity.

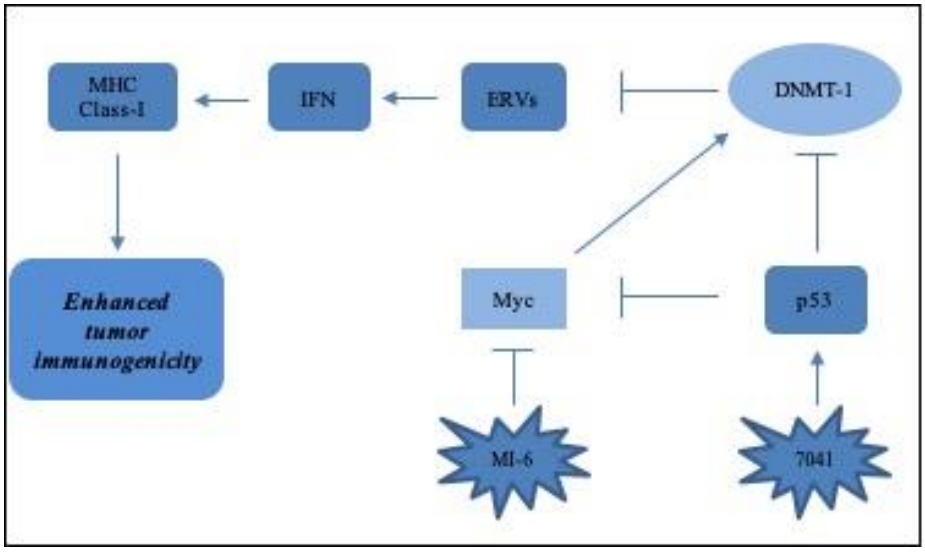

Fig. 15: A proposed model depicting how p53-activator and Myc-inhibitor regulates tumor responses to host immunity

7. Conclusion

Ablating the DNA methyltransferase 1 (DNMT-1) pharmacologically (using combination of p53-activator, ATPS-7041 and N-Myc inhibitor, MYCMI-6) enhances tumor immunogenicity by stimulating endogenous retrovirus expression and Interferon signaling.

Given the general role of p53-activator and Myc-inhibitor in regulating dsRNA and IFN responses, targeting both in combination may prove to be a universally applicable new strategy to overcome resistance.

Further work is needed to explore the function of p53 and Myc in immune cells. Moreover, further experiments need to be carried out for absolute confirmation.

8. Future perspective

Subsequent steps in our project could be performing of an enzyme-linked immunosorbent assay (ELISA) to further confirm IFN-β production. MHC class-I expression and the dsRNA stress could also both be further studied using Fluorescence-activating cell sorting analysis (FACS). The gold standard anti-dsRNA monoclonal antibody J2 for detecting the dsRNA could be accomplished to conclude both FACS and qPCR results.

Furthermore, knocking-out of p53 using CRISPR/Cpf1 system in SH-SY5Y cell line shall be carried out to prove p53 dependency, as confirmed in IMR-32 cell line.

9. Acknowledgement

First, I would like to express my sincere and tremendous appreciativeness to my supervisor **Galina Selivanova** for her ultimate guidance, motivation and support, especially with the ongoing pandemic by her so-grateful-for efforts to make sure we all stay healthy, safe and get through such a hard time in the most productive and efficient way possible. Thank you for pushing us forward in such times, and for always being there for advice, on both professional and personal level, and most importantly for being a professional fair-minded supervisor. Thank you for all the Fika we had, the delightful Christmas lunch and dinner, and the enjoyable conversations we had during. I would always be immensely grateful for giving me such an opportunity.

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إلي عيلتي، تيتة، خالتو، خالو، شكراً علي دعمكم المستمر ليا. اشوفكوا قريبا.
I love you!

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